GHENT UNIVERSITY
FACULTY OF PHARMACEUTICAL SCIENCES

BIODEGRADABLE DEXTRAN AND PEG MICRO- AND NANOGELS: PHYSICOCHEMICAL PROPERTIES AND SUSTAINED DRUG DELIVERY FEATURES

BIODEGRADEERBARE DEXTRAAN EN PEG MICRO- EN NANOGELS: FYSICOCHEMISCHE EIGENSCHAPPEN EN VERLENGDE VRIJSTELLING VAN GENEESMIDDELEN

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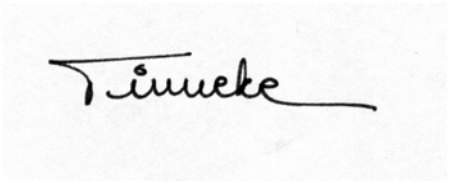

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ABBREVIATIONS

✳-PEG-HEMA	Star shaped PEG-HEMA
^{1}H-NMR	Proton Nuclear Magnetic Resonance
AFM	Atomic Force Microscopy
BMP	Bone morphogenetic protein
BSA	Bovine Serum Albumin
CDI	1,1'-carbonyldiimidazole
CHEMS	Cholesteryl hemisuccinate
CHOL	Cholesterol
CLSM	Confocal Laser Scanning Microscope
CS	Chitosan
CyA	Cyclosporine A
dex-GMA	Glycidyl methacrylate derivatized dextran
Dex-HEMA	Dextran hydroxyethyl methacrylate
DLS	Dynamic Light Scattering
DMAEMA	Dimethyl aminoethyl methacrylate
DMAP	4-N,N-dimethylaminopyridine
DMEP	4'dimethyl-epipodohyllotoxin
DMSO	Dimethyl sulfoxide
DOPC	1,2-dioleoyl-3-trimethylammonium propane chloride
DOPE	1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine
DOTAP	1,2-dioleoyl-3-trimethylammonium propane chloride
DPPC	1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
DPPG	1,2-dipalmitoyl-*sn*-Glycero-3-[phospho-*rac*-(1-glycerol)
DPPlsC	1,2-di-O-(Z-1'-hexadecenyl)-*sn*-glycero-3-phosphocholine
DSPE-PEG$_{2000}$	1,2-Distearoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-2000] (Ammonium Salt)
DS	Degree of Substitution
EPR	Enhanced permeability and retention
FITC	Fluorescein isothiocyanate
FU	Fluoroacil
GPC	Gel Permeation Chromatography

HCPT	10-hydroxycamptothecin
HEMA	Hydroxyethyl methacrylate
HPLC	High Performance Liquid Chromatography
HPMP	hydroxypropyl methylcellulose phthalate
HQM	Hydroquinone monomethyl ether
I2959	Irgacure 2959
KPS	Potassium peroxodisulfate
KS	Kaposi Sarcoma
MAA	Methacrylic acid
MBA	N,N'-methylene bis-acrylamide
MWCO	Molecular Weight Cut Off
PACA	Poly(alkyl cyanoacrylate)
PB	Phosphate buffer
PBCA	Poly(butyl cyanoacrylate)
PBP	Poly(butyl pyrolidone)
PCL	Poly(ε-caprolactone)
PCLLA	Poly(caprolactone-co-lactide)
Pd	Polydispersity
PECA	Poly(ethylene-2-cyanoacrylate)
PEG	Poly(ethylene glycol)
PEG-HEMA	Poly(ethylene glycol) hydroxyethyl methacrylate
PEG-lac-HEMA	PEG-lactate-HEMA
PLA	Poly(lactide)
PLGA	Poly($_{DL}$-lactide-co-glycolide)
PVA	Poly(vinyl acetate)
PVP	Poly(vinylpyrrolidone)
RES	Reticuloendothelial system
rho	Rhodamine
SOPC	1-Stearoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine
TEM	Transmission Electron Microscope
TEMED	N,N,N',N'-tetramethylenediamine
TPGS	d-α-tocopheryl poly(ethylene glycol)$_{1000}$ succinate
TPP	Tripolyphosphate

TX 100	Triton X 100
VEGF	Vascular endothelial growth factor

SYMBOLS

τ	Tensile strength
η	Dynamic Viscosity
ζ	Zèta potential
π_{el}	Elastic pressure
π_{osm}	Osmotic pressure
ΔP	Pressure difference
π_{sw}	Swelling pressure
$[\eta]$	Intrinsic viscosity
a	Activity
c^*	Overlap concentration
d_h	Hydrodynamic diameter
G’	Elastic modulus
M_n	Number average molecular weight
M_w	Weight average molecular weight
r	Radius
S	Absorbance decrease per minute
v	Partial specific volume
w	Weight
ρ	Density

GENERAL AIM OF THIS THESIS

The last decade there is a booming development of therapeutic molecules with promising results to treat a wide variety of inherited and acquired diseases. pDNA, DNA, RNA, siRNA, peptides, proteins… are modern drug candidates, but often they lack stability once entered in the human body. Most of these molecules are destabilized by enzymatic attack or they are cleared from the bloodstream by phagocytosis before they can carry out any therapeutic intervention. As a result, there is a growing interest in drug carriers which can deliver therapeutic molecules intracellularly, intact and at the desired place and time.

The general aim of this study is to develop nanometer sized hydrogel particles which are able to load therapeutic molecules, deliver them inside the cell in a time controlled manner, with high efficiency and without loss of the molecules' integrity. This process is schematically represented in Figure 1.

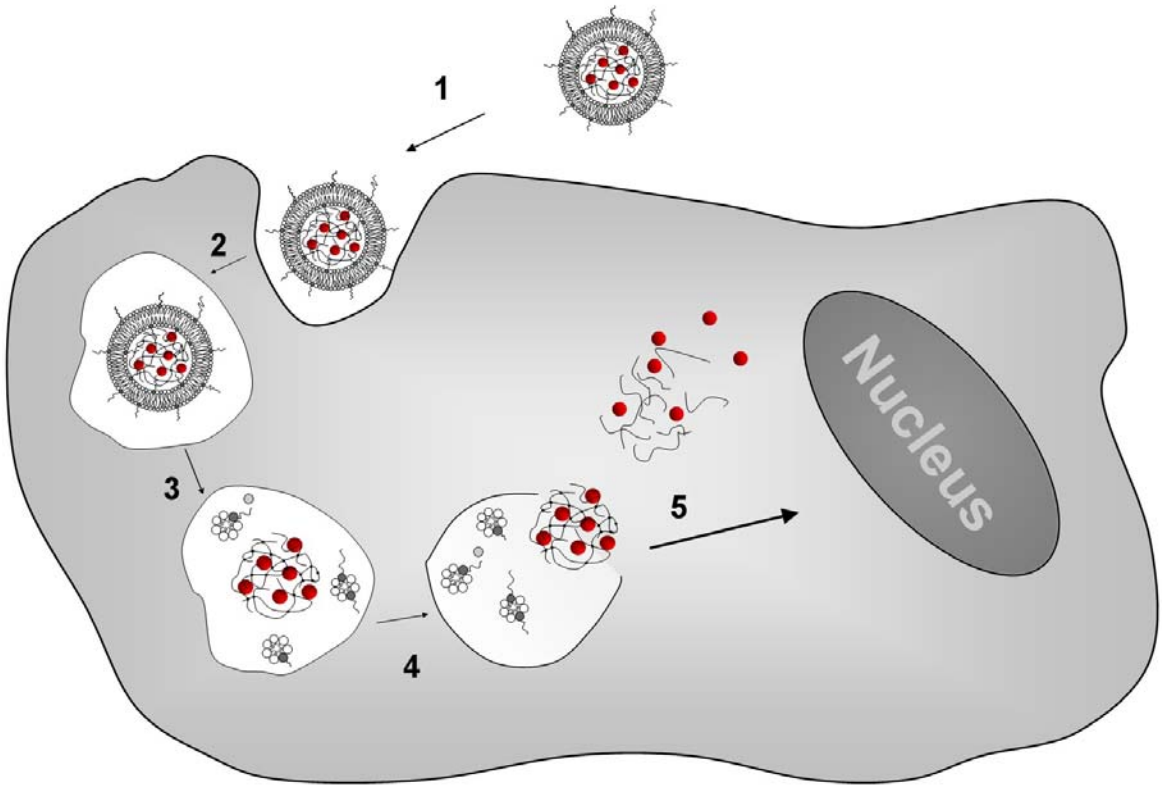


Figure 1. Schematic overview of intracellular uptake of PEGylated and pH sensitive lipid coated nanoparticles which release their coating upon acidification in the endosome. As a result, the particles easily escape and release their therapeutic content in the cytosol. (1) engulfment of the PEGylated lipid coated particle; (2) the particle is enclosed in an endosome; (3) degradation of the PEGylated lipid coating; (4) endosomal release of the particle into the cytosol; (5) delivery of the therapeutic content.

The first key aspect of this thesis includes the synthesis of nanoscopic dextran nanogels with and without a surrounding lipid-coating. Our first aim is to achieve intracellular delivery, so the particles must be taken up by cells. The second aim was to provide sustained delivery of therapeutic components, so the nanogels should contain some model molecules and release them in a time controlled manner. As stability during blood circulation is a main feature in drug delivery, the particles must be provided with a PEGylated layer as it is well known that this creates a steric barrier to avoid macrophage uptake. On the other hand, it is known that PEG hampers the endosomal escape of particles after cellular uptake. Keeping this in mind, it would be convenient to dispose of nanogels that have a PEGylated lipid layer that comes off once entered in the endosome. The intrinsic low pH of the endosomes will be the ideal trigger to achieve this.

The second key aspect is the evaluation of poly(ethylene glycol) for the formation of drug delivery systems. It is important to gain insight in the osmotic swelling behavior of PEG for the production of “self-exploding microparticles”, i.e. microscopic hydrogel particles surrounded by a lipid coating showing pre-programmed pulsed drug delivery. Different polymerizable PEG components, like PEG-HEMA, PEG-lactate-HEMA and star shaped PEG-HEMA were evaluated for microscopic as well as nanoscopic drug delivery device.

STRUCTURE OF THIS THESIS

Chapter 1 represents a literature overview on "sustained release from nanosized matter".

Chapters 2-4 describe the production of biodegradable nanoparticles for the controlled release of therapeutic molecules with intracellular target. In **chapter 2** the synthesis of nanogels using liposomes as a nanoscopic reaction container is described. Liposomes were filled with a polymerizable dextran (hydroxyethyl methacrylated dextran, dex-HEMA) and subsequently irradiated with UV. As a result, lipid-coated nanogels can be produced. By adding a detergent, the lipid-coating can be removed and naked nanogels are obtained. As it is known that dex-HEMA is biodegradable, it was checked whether nanosized dex-HEMA hydrogels with tunable degradation properties can be obtained. We also wondered whether these particles are taken up by cells.

Having established a good procedure to prepare biodegradable dextran nanogels in chapter 2, these nanogels were loaded with some model proteins in **chapter 3**. The loading efficiency is determined and we also wondered whether the encapsulated proteins maintain their biological activity. As our aim is to design nanogels showing sustained drug delivery profiles, we tried to govern the release profile of the included molecules in function of time by altering the degree of substitution of the nanogels. The particles' stability in serum was evaluated and also the possibility to freeze-dry them with an eye to long-time storage.

The presence of the lipid-coating offers possibilities to change the surface properties of the nanogels. In **chapter 4** we inserted PEGylated lipids into this coating in order to increase their serum stability. It was also checked whether the particles are still taken up by cells. As PEGylation often hampers endosomal escape we also wanted to design nanoparticles with a PEGylated lipid-coating that comes of the particles at endosomal pH. This was achieved by insertion of a synthetically derived diplasmenylcholine.

In **chapter 5 and 6** we took a close look at the properties and possibilities of poly(ethylene glycol) (PEG) in the field of drug delivery. In **chapter 5** the degradation behavior of hydroxyethyl methacrylated poly(ethylene glycol) (PEG-HEMA) was compared with dex-HEMA. The osmotic swelling behavior of both polymers in the presence of unattached PEG and dextran chains was examined. This insight is of great importance in the design of self-rupturing microcapsules.

Chapter 6 screens different polymerizable PEG as hydrogel forming polymers. First, the degradation behavior of PEG-HEMA, PEG-lactate-HEMA and star shaped PEG end capped with HEMA (✳-PEG-HEMA) was evaluated on the macroscopic scale, showing that a time scale ranging from hours to months can be

achieved. Second, lipid-coated and naked microgels were produced and the release profile of a fluorescent molecule was mapped. In the last part of this chapter PEG-HEMA nanogels (with and without lipid-coating) were produced following the procedure described in chapter 2. BSA was inserted and the release profile of this protein was followed in function of time.

1

SUSTAINED RELEASE FROM NANOSIZED MATTER

Abstract

The last decade a significant amount of active macromolecules like peptides, proteins and oligonucleotides is developed in the combat against a wide range of diseases. However, at this moment some problems like stability, toxicity and poor biodistribution block the way to daily clinical use. Lots of efforts have been performed to obtain suitable carrier systems for these macromolecular drugs. Nanoscopic particles show great promises to this end. As they encapsulate the drug molecules in their interior, they keep them protected from enzymatic attack and they provide means for controlled and targeted delivery to specific organs or cells. This first chapter outlines the concepts that have been proposed to release drugs in a sustained manner from nanoscopic delivery devices.

INTRODUCTION

There is still a quest for an appropriate system for parenteral administration of macromolecular drugs, such as proteins, oligonucleotides or DNA. Although these drugs have promising capacities, some non-ideal properties, such as poor solubility, rapid breakdown *in vivo,* rapid clearance by the reticuloendothelial system (RES), poor biodistribution and lack of selectivity for target tissues[1] hinder their entrance in daily clinical use. Nevertheless, a lot of these problems can be overcome by the use of an appropriate drug delivery system. Pharmacokinetics can be modified in the first place by a change in molecular structure of the drug itself, e.g. attachment of PEG moieties to the therapeutic compound. Another possibility that is more often used is by a change in drug formulation, more precisely by encapsulating the drugs in nanospheres or microspheres. Due to their size micrometer sized particles cannot be used for systemic diseases -such as cancer-, as blood circulation of the delivery system is required. Therefore, they are not discussed in this overview.

In this first chapter an overview is given of the literature describing nanoscopic drug delivery systems showing sustained release for a prolonged period of time. Liposomal constructs, nanoscopic hydrogels, surface modified nanoscopic particles and core shell type nanoparticles showing sustained release for a *prolonged* time (i.e. more than 24 hours) were discussed.

LIPOSOMES

Introduction

Liposomes are naturally occurring nanoscopic structures, consisting of one or more concentric lipid layers, enclosing an aqueous solution as represented in Figure 1. They can act as sustained release delivery system, as they can entrap thousands of (small) molecules[2], offering possibilities for the encapsulation and sustained release of both lipophilic and hydrophilic components, as the molecules can be retained inside respectively the lipid layers or the central cavity of the liposome. Besides, due to their size they are passively targeted to the organs of the RES.

Liposomes are relatively stable, biocompatible and biodegradable and prolonged drug release is achieved as a result of the diffusion of the drug through numerous phospholipid membranes[3]. Since their first description in 1968[4], liposomes are described extensively as drug delivery system for therapeutics against a wide range of diseases.

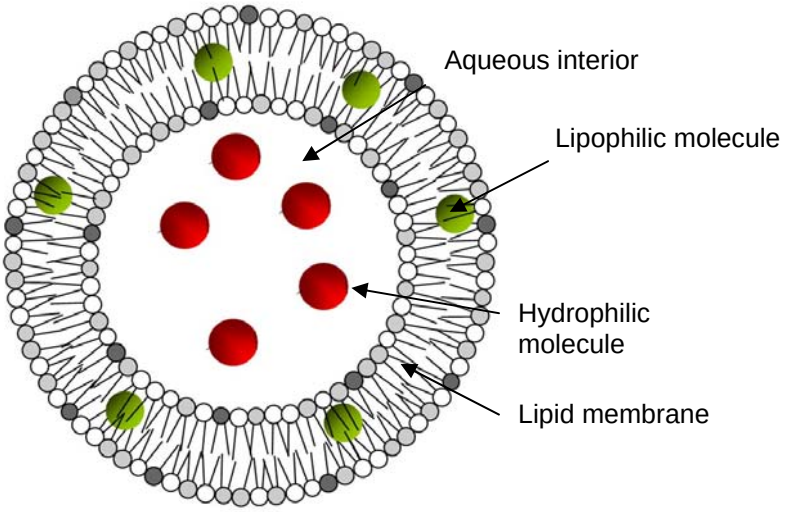


Figure 1. Schematic representation of a liposome containing both hydrophilic and lipophilic molecules

Liposomal constructs in the combat against a wide range of diseases

Conventional liposomal formulations of anthracyclines have already been approved for clinical use in AIDS-related Kaposi's sarcoma (Daunoxome)[5] and breast **cancer** (Myocet)[6]. Other anti-cancer drugs, as mitoxantrone[7] and vincristine[8;9] show better biodistribution patterns when they are administered in the liposomal form.

Also, liposomes are intensively studied in the combat against mycobacterial infections, such as **tuberculosis and leprosy**. As mycobateria are intracellular pathogens the drug has to be delivered inside the cell and this for an extended period of time. There are number of reports available that used conventional liposomes for the treatment of *Mycobacterium avium* complex and *M. tuberculosis* infections using various drugs such as streptomycin[10], amikacin[11], gentamycin[12] and kanamycin[13]. Due to the sustained release profile, the liposomal drugs were administered with a limited number of injections, resulting in improved pharmacokinetic properties, minimum toxicity and better patient compliance.

Recently, Ishida et al.[14] showed that fasudil, a cerebral vasodilator against **cerebral vasospasm** that can cause severe side effects when administered in the free form, can be given to rats in the liposomal form and causes no significant changes in values for mean arterial blood pressure, heart rate or arterial carbon dioxide pressure. The release of fasudil from the liposomes was caused by the diffusion of fasudil molecules through the lipid membranes, not by the disruption of liposomes. Animals treated with liposomal fasudil showed significantly improved neurological outcomes after 24h observation period compared to the control group.

Yang et al.[15] used liposomal interferon-α-2b for the treatment of **genital herpes**. Here the liposomes are filled with high encapsulation efficiency (>80%) and they are administered intramuscularly. The liposomes (<100 nm) act as a sustained reservoir at the topical site of injection and provide stabilization of the drug during *in vivo* transport and in blood circulation. They are targeted to RES organs such as the

liver and spleen. In this way interferon-α-2b can keep a higher topical concentration for a prolonged period and interferon must be administered less frequently.

Nanoscopic liposomes are not only suitable for parenteral injection, but also - and especially- for **eye treatment**: small particles are necessary for delivery to the inner compartment of the eye. Intravitreal injection in the eye should be applied as less as possible as severe risks for infection occur, so also here sustained release from liposomes could offer benefits. Due to the sustained release liposomes encapsulating conventional small molecular weight drugs (usually anticancer) and larger genetic drugs (oligonucleotide and plasmid DNA) could be administered by intravitreal injection with significantly increased drug half-life and decreased intraocular side-effects and higher drug levels at the target site[16].

Disadvantages related to the use of liposomal constructs

Although at the present time, liposomes are the premier particulate carrier systems over other drug delivery systems in terms of safety and usage in the clinic, liposomes may not be the best option for long term delivery of drugs, as liposomal particles are rapidly sequestered into the liver, spleen, kidneys and RES, it is more difficult to entrap macromolecular drugs with high efficiency[17] and the included molecules are released quickly in comparison with polymeric delivery systems and controlling the release rate is not straightforward. Besides, large scale manufacture of sterile liposomes is expensive and technically complicated[18].

NANOGELS

Introduction

Hydrogels are network structures formed by polymers that are mutually attached by cross-links, keeping large amounts of water in the formed matrix. From a biomedical point of view, hydrogels have gained much attention last decades as they are considered to be attractive delivery systems for a wide range of therapeutic molecules due to some unique properties. They can be loaded with high amounts of molecules (as the cross-links keep them entrapped in the formed matrix) and the release of the molecules can be controlled by varying the hydrogels' characteristics.

The following part of this first chapter handles on nanoscopic hydrogel particles in which therapeutic molecules are embedded in the hydrogel matrix (as represented in Figure 2), this in contrast to nanoparticles which arise through self-assembling of DNA and degradable cationic polymers. These so-called polyplexes are widely described in literature and are thus not the scope of this overview.

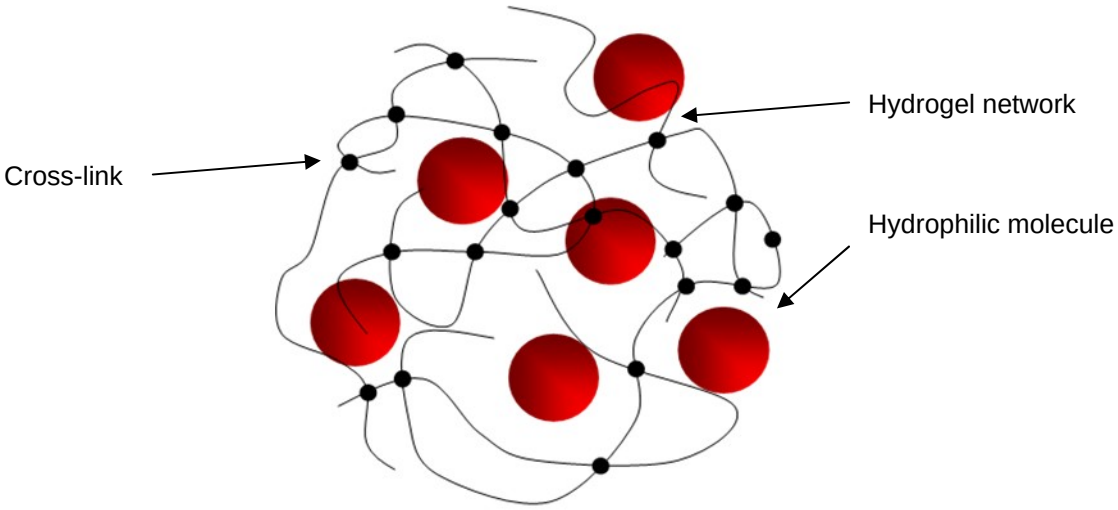


Figure 2. Schematic representation of a nanoscopic hydrogel containing therapeutic molecules

To make a chance as drug delivery system, the nanogels should fulfill some basic requirements: (1) they should encapsulate the therapeutic molecule intact and with a high efficiency. In the production process of nanogels harming activities, such as polymerization, sonificiation or contact with organic solvents, can damage the encapsulated drugs and must be evaluated carefully. (2) *In vivo*, the polymer matrix must protect the drugs against aggregation with serum components or enzymatic attack. (3) The degradation of the matrix should not generate any hostile internal environment, for example a drop in pH. (4) For toxicity reason, burst release should be avoided.

Nanogels can be categorized in many possible ways. (1) The way of cross-linking used to form the hydrogel: in comparison with chemical cross-linking, physical cross-linking is in general less permanent and based on electrostatic or hydrophobic interaction or hydrogen bonding. (2) The nanogels' reaction on environmental changes can also be used as a classification criterium. Non-responsive hydrogels, as the name already suggest do not react on environmental changes. Stimuli responsive hydrogels can react on light, pH, temperature, ionic strength, electric field or the presence of biomolecules (like e.g. enzymes). (3) Another possible way of classification is the production method of the nanogels. Commonly used methods are emulsification/solvent evaporation, emulsification/solvent diffusion, solvent displacement and interfacial deposition or salting-out[19], but also more sophisticated techniques can be used, like using liposomes or reverse micellar droplets as nanoreactors to form the nanogels[20;21]. (4) A last possible way of classification is following the method of loading the particles with the desired drug molecules. This loading can be performed in two ways: the first is the loading during the preparation of the particles. Mostly, the drugs are added during the formation process, but this has the main disadvantage that the therapeutic molecules are exposed to harming conditions like sonificiation, polymerization, contact with organic solvents… Another possible way is the *a posteriori* method: the particles are uploaded *after* their formation. Most often, electrostatic interaction is involved in this method: charged

particles are loaded with oppositely charged molecules. The main disadvantage of this system is that the drug molecules are attached more likely at the outside of the particles and as a result, they are more susceptible to enzymatic attack in the body and they are also released more easily, often resulting in a burst release.

In the following paragraph we will classify the nanogels according to their composing material. This can range from synthetic polymers to naturally occurring polysaccharides.

Synthetic polymers as nanogel forming material

The first polymers that were used for the sustained release of therapeutic molecules were poly(acrylamide), poly(vinyl alcohol) and poly(hydroxyethyl methacrylate)[22;23], but due to toxicity reasons, research was focused more on other synthetic polymers.

Bharali et al.[24] used **poly(vinylpyrrolidone)** (PVP, Figure 3), a hydrophilic, biocompatible and non-antigenic polymer to prepare injectable hydrogel nanoparticles with reverse micellar droplets as nanoreactors and N,N'-methylene bis-acrylamide (MBA) as a cross-linking agent. FITC-dextran was used as a model hydrophilic drug and its release profile could be controlled by changing the amount of cross-linker, the loading of the dye and the pH of the external medium. That way, release times ranging form a few days to almost a month could be achieved. As the degradation behavior of these PVP-MBA particles is almost zero, it is believed that the release is a diffusion controlled process from the highly swollen nanoparticles[25].

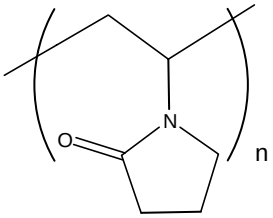
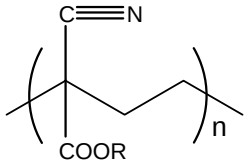


Figure 3. Chemical structure of poly(vinylpyrrolidone) (PVP).

These particles could also be used to entrap allergens of *Aspergillus fumigatus*, a complex mixture of more than 30 allergenic and antigenic proteins used for vaccination. *Aspergillus fumigatus* is one of the most common Aspergillus species to cause diseases in immunocompromised individuals. Release profiles of the allergens out of the nanoparticles ranging from two weeks up to 10 weeks could be achieved. As a result, in mice subcutaneous injected nanogels with entrapped antigens show sustained levels of IgG antibodies, showing that the nanogels act as a repository for the antigens, protecting them from rapid degradation and allowing small antigenic mass to be delivered continuously and effectively. The IgG levels achieved with three divided doses of entrapped antigen could also be achieved with same amount of entrapped antigens administered in a single dose[26].

However the major disadvantage of these delivery systems is that they are not biodegradable. As a result, the risk of chronic toxicity due to intracellular or tissue overloading of non-degradable polymers can be considered as a limitation for systemic administration.

Nanoparticles based on biodegradable polymers such as **poly(alkyl cyanoacrylate)** (PACA, Figure 4), are being extensively investigated as delivery systems for small drug molecules[27-33], proteins[23;34-36] or nucleic acids[37]. PACA nanoparticles can protect the active molecules and release is controlled by erosion of the particles.



PMCA: poly(methyl cyanoacrylate) R: CH_3
PECA: poly(ethyl cyanoacrylate) R: CH_2CH_3
PBCA: poly(n-butyl cyanoacrylate) R: $CH_2CH_2CH_2CH_3$
PIBCA: poly(iso-butylcyanoacrylate) R: $CH_2CH(CH_3)_2$
PHCA: poly(isohexyl cyanoacrylate) R: $CH_2CH_2CH_2CH(CH_3)_2$

Figure 4. Chemical structure of different poly(alkyl cyanoacrylates)

However, for sustained release, most of the poly(alkyl cyanoacrylate) nanoparticles release their content too quick, speaking in terms of a few hours. Xun et al.[38] tried to overcome the problem of fast release of 10-hydroxycamptothecin (HCPT) out of poly(butyl cyanoacrylate) (PBCA) nanogels by associating the particles with poly(butyl pyrrolidone) (PBP). As a result, the release rate was significantly decreased: *in vitro* it was shown that 75% of the initially loaded HCPT was released after 48h. In comparison, without the presence of PBP 70% of HCPT was released within 10h from the same PBCA particles. Normally, HCPT as such is metabolized rapidly *in vivo*. Nevertheless after injection of HCPT loaded PBCA nanoparticles associated with PBP, the HCPT plasma concentration decreased rapidly in the first 2h and went up slightly afterwards and maintained the same level for 30h, suggesting a sustained release of HCPT from the PBP associated PBCA nanoparticles. A possible reason was that the nanogels were taken up by the RES organs, which resulted first in a rapid decrease in blood concentration. Afterwards HCPT was slowly released from the nanogels in the liver or spleen so that the blood concentration rose slightly.

Zhang et al.[39] used PBCA nanogels to deliver mitoxantrone, an anti-neoplastic agent. The *in vitro* drug release data show that there are two phases in the drug release profile: first a burst release of more than 50% the first 24h followed by a slower release for 13 days. The anti-tumor effect of an intravenous injection of mitoxantrone loaded in these particles turned out to be better when compared to an

injection of a mitoxantrone solution: the inhibition of orthotopically transplanted human hepatocellular carcinoma cells in rats was higher and the acute toxicity was lower. The latter was due to the direct accumulation of the mitoxantrone to the liver when it was loaded into the PBCA particles.

However, PACA's did not seem to be completely harmless, as during degradation formaldehydes are formed. It was observed from cell culture experiments that the viability of the cells was higher with a lower release of formaldehyde. Alkyl homologues such as ethyl cyanoacrylate revealed a higher tissue toxicity than higher alkyl homologues[40].

Very recently, Devarajan et Sonavane[41] formulated **Eudragit** (a co-polymer composed of acrylic acid and methacrylic acid) nanoparticles containing gliclazide, an oral hypoglycemic agent widely used for the treatment of non-insulin-dependent diabetes mellitus, to achieve a sustained drug delivery system suitable for *per oral* administration. *In vitro* studies revealed that the release could be extended for over 24h, depending on the pH of the release medium and the surfactant used during production. *In vivo*, the encapsulated gliclazide showed a sustained activity in diabetic rat model as compared to plain gliclazide. As a result, the dose frequency can be reduces, side effects are decreased and the patient compliance is improved.

Poly(lactide) (PLA) and **poly(lactide-co-glycolide)** (PLGA, Figure 5) are probably the most studied biodegradable matrices for sustained drug delivery from nanoscopic materials. It has been approved by the US Food an Drug Administration for application in drug delivery[42], it is GMP grade, commercially available and it has a long history of safe use in both medical applications and drug delivery. PLGA has been successful as a biodegradable polymer because it undergoes hydrolysis in the body to produce the composing monomers, being lactic acid and glycolic acid. These two monomers under normal physiological conditions, are by-products of various metabolic pathways in the body and as a result are completely non-toxic, they are eliminated from the body by the Kreb's cycle. Depending on their composition and molecular weight, PLGA provides degradation rates ranging from months to years[43].

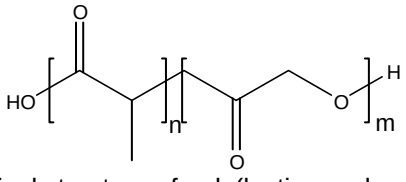


Figure 5. Chemical structure of poly(lactic-co-glycolic acid) (PLGA)

PLGA nanoparticles can be used for the delivery of a wide range of therapeutic molecules to intracellular targets. The intracellular destination of the particles depends mostly on the particles' surface charge: nanoparticles which show transition in their surface charge from anionic (at pH 7) to cationic in the acidic

endosomal pH (pH 4-5) were found to escape the endosomal compartment whereas the nanoparticles which remain negatively charged at pH 4 were retained mostly in the endosomal compartment[44].

Already in 1999, Labhasetwar et al.[45] were successful in encapsulating plasmid DNA (pDNA) into nanospheres and sustained release of the encapsulated pDNA *in vitro* under physiological conditions for over 17 days was demonstrated. In cell culture, gene transfection with model genes like fire fly luciferase and heat stable human placental alkaline phosphatase using nanospheres was up to five orders of magnitude greater than the gene expression with the same amount of pDNA in solution. Also *in vivo,* in a rat bone osteotomy model sustained gene expression was observed in tissue retrieved from the gap five weeks after the surgery. This gene delivery strategy could be used to facilitate bone healing using therapeutic genes such as bone morphogenic protein. Cohen et al.[46] showed sustained marker gene expression with pDNA containing PLGA nanoparticles both *in vitro* and *in vivo* for up to 28 days.

PLGA nanoparticles were also prepared for oral drug delivery to the inflamed gut tissue in inflammatory bowel disease. Rolipram, an anti-inflammatory model drug, was incorporated within PLGA nanoparticles and showed a sustained release *in vitro* for over 1 week resulting from the diffusion of the drug through the polymer. These particles were administered to rats once a day orally for five consecutive days. During the next 5 days when animals were kept without drug treatment, the drug solution group displayed a strong relapse, whereas the nanoparticles groups continued to show reduced inflammation levels[47]. Another oral drug delivery device composed of PLGA was proposed by Mittal et al[48]. They studied the oral bioavailability and sustained release of estradiol, one of the most potent natural estrogens. *In vitro,* drug release decreased with increase in molecular weight of the PLGA and the lactide content. The *in vivo* performance of the nanoparticles was assessed in rats and showed that with all the PLGA nanoparticles the same dose produced detectable blood levels for 5-11 days, depending on the molecular weight, the polymer composition and the particles size. This was a remarkable improvement, as the pure drug could only be detected in the blood for 1 day. Derakhshandeh et al.[49] used PLGA nanoparticles for oral delivery of 9-nitrocamptothecin. The release of this anticancer agent *in vitro* could be maintained for up to one week.

As already mentioned above, nanoscopic particles are particularly suited for delivery of drugs to the inner compartments of the eye. Aukunuru et al.[50] used PLGA nanoparticles for the sustained delivery of vascular endothelial growth factor (VEGF) antisense RNA to retinal cells. Retinal neovascular diseases including diabetic retinopathy, retinopathy of prematurity and age-related macular degeneration, are typically associated by invitreal elevated VEGF levels. Antisense oligonucleotides are single-stranded pieces of nucleic acids with a sequence specifically designed to bind a target sequence of intracellular RNA blocking the expression of a target protein, like in this case VEGF. Thus, VEGF antisense RNA can inhibit VEGF production to treat retinal neovascular diseases. VEGF antisense oligonucleotides

encapsulated into PLGA nanoparticles were released very slowly: the cumulative oligonucleotide release from the particles at the end of day 10 was only 39%. The cellular uptake of oligonucleotides was significantly increased when entrapped in nanoparticles. Moreover these nanoparticles significantly reduced VEGF mRNA levels and VEGF protein secretion from RPE cells, suggesting that the antisense oligonucleotides were able to reach intracellular compartments at VEGF inhibitory concentrations. Kompella et al.[51] successfully prepared PLA nanoparticles loaded with budesonide, a new agent capable of inhibiting VEGF expression in retinal pigment epithelial cells through its glucocorticoid receptor affinity. Budesonide can be encapsulated with high efficiency and drug loading (resp. 65% and 16.25%) and *in vitro* sustained release at the end of two weeks was approximately 50%. *In vivo* 7 days after subconjunctival administration, sustained retinal budesonide levels were achieved when compared with a solution form in a rat model.

The problem with PLGA is that it is often too hydrophobic, degradation rate is too slow, resulting in too slow drug release and often chemical emulsifiers like poly(vinyl acetate) (PVA) were used during synthesis. The latter polymer has been found to show disadvantages including low emulsification efficiency, side effects and difficulties to wash away in the formulation process. To cope with these problems, Zhang et al.[52] incorporated D-α-tocopheryl poly(ethylene glycol)$_{1000}$ succinate (TPGS) in PLA instead of glycolide. The result is a copolymer, PLA-TPGS (Figure 6A) from which nanoparticles can be made (Figure 6B) with improved drug encapsulation efficiency and enhanced cellular uptake when compared with PLGA. The study shows that PLA-TPGS nanoparticles can be loaded with paclitaxel and complete drug release takes much longer time than 30 days, depending on the molecular ratio PLA/TPGS used in the composing polymer: after 30 days, the accumulative drug release approaches 55-65%. In comparison, the drug release from PLGA nanoparticles within one month is usually 30-40%, which is too slow to meet therapeutic needs. The novel PLA-TPGS nanoparticle formulation of paclitaxel showed significant advantages in achieving larger cytotoxicity and smaller IC_{50}, over the commercial formulation of paclitaxel (Taxol®, a formulation with Cremophor EL).

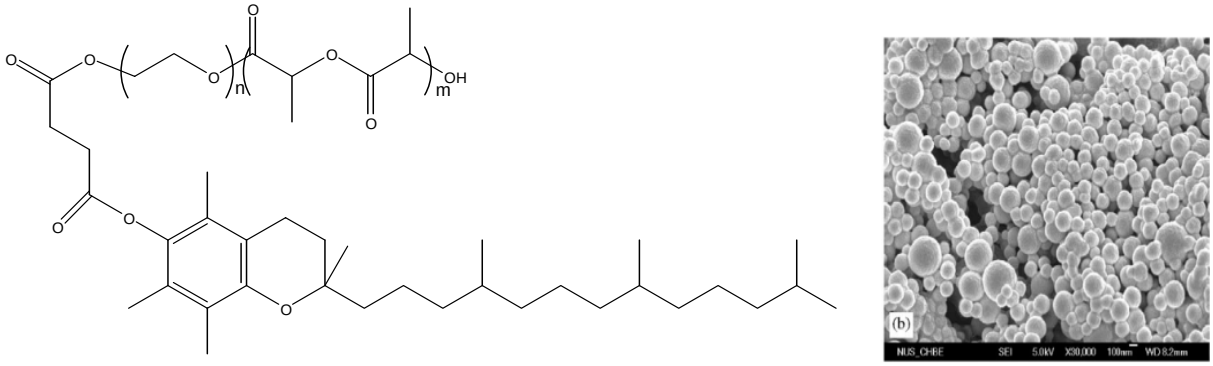


Figure 6. (A) Molecular structure of PLA–TPGS copolymer and (B) SEM image of paclitaxel-loaded PLA–TPGS nanogels (84:16 molar ratio)[53]

As already briefly mentioned above, PLGA shows some drawbacks to be used as a drug delivery system like hydrophobicity and slow degradation. In addition, it is well shown that degradation of PLGA results in a decrease in pH in the particles due to the formation of lactic acid and as a result, acid sensitive molecules can be damaged during encapsulation, although basic additives such as $Mg(OH)_2$ and $Ca(OH)_2$ can avoid this[54]. Also, it was demonstrated *in vitro* that the so called "bioinert" PLA can be cytotoxic to immune cells: cells that had phagocytosed PLA particles showed increased signs of cell damage and administration of "predegraded" PLA particles resulted in necrotic cells and cell debris after three or four days[55]. Besides, different factors influence the release rate out of PLGA nanoparticles: not only the chemical composition of the PLGA but also the molecular weight, particle size, morphology and processing condition influence the release. For example, PVA is most commonly used as an emulsifier during the production process of PLGA nanoparticles. It is shown that a fraction of PVA remains associated at the nanoparticle surface and affects the physical and cellular uptake properties of nanoparticles. As a result, it is difficult to predict the *in vivo* release characteristics[56]. Bala et al.[57] performed research on the influence of stabilizers used during processing, like PVA, didodecyldimethylammonium bromide (DMAB) and a combination of PVA and chitosan (PVA-CS) on the release characteristics of ellagic acid, a photochemical with antioxidant properties. Release was depending on the hydrophobicity of the stabilizer following the order PVA > PVA-CS > DMAB releasing respectively 50%, 38% and 24% over 6 days[57].

Polysaccharides and derivatives as nanogel forming materials

While synthetic polymers have the advantage of sustaining the release of the encapsulated therapeutic agent over a period of days to several weeks they are in general limited by the use of organic solvents and relatively harsher formulation conditions. Natural polysaccharides constitute an important class of physiological materials, displaying well-documented biocompatibility and biodegradability, which are the basic characteristics for polymers used as a compound for drug delivery systems.

Alginate is a naturally occurring polysaccharide composed of mannuronic acid and gluconic acid (Figure 7) and because of the negative charge of the carboxylic groups from the uronic acid, it can be cross-linked into nanoscopic structures by electrostatic interaction with oppositely charged counter ions[58]. Is has been extensively investigated for drug delivery and tissue engineering applications[59;60], but the use on the nanoscopic scale is rather rare.

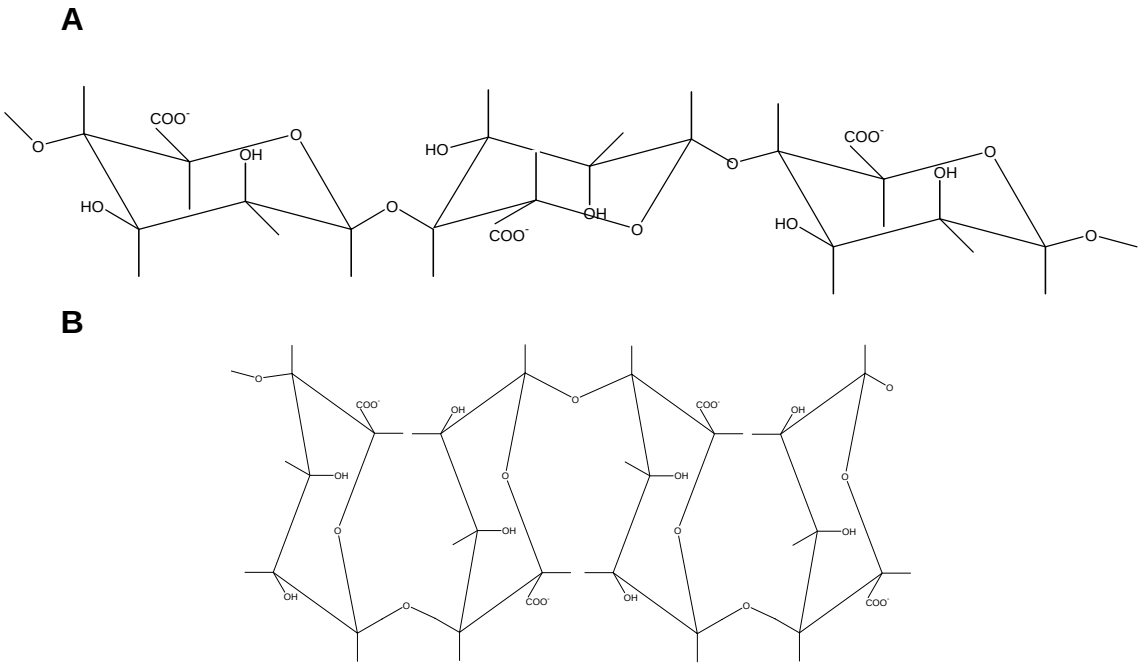


Figure 7. Chemical structure of (A) Poly(mannuronic acid) and (B) poly(guluronic acid). The negative charges of the carboxylic acids can interact with oppositely charged molecules to form nanoscopic hydrogels.

However, Pandey et al.[61] showed that it is possible to have a slow and controlled release of antifungal drugs, clotrimazole and econazole, by encapsulating them into alginate nanoparticles stabilized with Ca^{2+} and chitosan. The formulations were orally administered to mice and pharmacokinetic data suggested that there was a controlled drug release for 5-6 days whereas unencapsulated drugs were cleared from the bloodstream within 3-4 hours.

Chitosan and its derivatives are probably the most used modified sugars for sustained release. It is a deacetylated product of chitin consisting of D-glucosamine and N-acetyl-D-glucosamine, comprising several benefits such as biocompatibility, biodegradability low immunogenicity and low cost[62]. Moreover, due to its cationic nature this polymer is mucoadhesive offering possibilities for transmucosal drug delivery and transiently opening the tight junction between epithelial cells[63]. It forms hydrogel nanoparticles by electrostatic interaction with polyanions like tripolyphosphate (TPP), simply by dropping chitosan droplets into a TPP solution. Calvo and co-workers[64] succeeded in maintaining *in vitro* release of tetanus toxoid for 18 days and of BSA for 8 days. Deng et al.[65] also used TPP as a cross-linking agent to encapsulate lysozyme. The release was in the range of days and could be controlled by the concentration of chitosan, TPP or initially added lysozyme or the molecular weight of the used chitosan.

Kim et al.[66] used a hydrophobically modified glycol chitosan. This amphiphilic polymer self-assembles in water forming nanogels. The particles can be loaded with paclitaxel, a hydrophobic antitumor drug, using a simple dialysis method. Currently paclitaxel is formulated with Cremophor EL, which is not an inert vehicle as it exerts a series of biological and physiological effects as nephrotoxicity and anaphylactic

reactions[67;68]. This newly developed chitosan based delivery system turned out to be less toxic. *In vitro* sustained release can be achieved for over 8 days (assessed in PBS at 37°C pH 7.4) while *in vivo* tumor growth in tumor bearing mice can be prevented for the same period. Cha et al.[69] also used glycol modified chitosan: they used succinic anhydride as a pH sensitive linker to introduce cisplatin, an anticancer drug. Nano-aggregates of 180-300 nm released cisplatin in a sustained manner: under physiological conditions, 40% of the incorporated cisplatin was released for 11 days. The pH sensitive linker *cis*-aconityl was also used to provide a pH sensitive bridge between glycol chitosan and doxorubicin[70;71]. This polymer can be used to produce nanoscopic hydrogels which are mainly directed to the kidneys, tumor and liver after systemic administration. Moreover, the drug content can be increased by an additional physical loading of free doxorubicin, resulting in a eight time higher loading capacity than the amount of chemically conjugated doxorubicin. The release of doxorubicin out of these nanogels at pH 7 was negligible for almost ten days. At endosomal pH however, a slow release was observed due to the pH sensitive linker.

Very recently, Chen et al.[72] developed novel composite nanoparticles based on glycidyl methacrylate derivatized **dextrans** (dex-GMA) combined with gelatin. The gelatin was added to load the particles with bone morphogenetic protein (BMP) as electrostatic interactions occurred between acidic BMP and basic gelatin segments. The *in vitro* drug release studies showed that BMP released from the particles could maintain more than 12 days in the presence of dextranase. This system could provide a proper local BMP delivery system for delivery of active drugs for periodontal healing enhancement.

Wang et al.[73] encapsulated cyclosporine A (CyA), a cyclic oligopeptide with potent immunosuppressive activity, with high yield and encapsulation efficiency into a **cellulose** derivative: hydroxypropyl methylcellulose phthalate (HPMP). HPMP is an enteric dissolved polymer from which nanoscopic particles can be obtained by the solvent displacement technique. It was shown that the concentration in the blood after oral administration of a single dose of CyA loaded HPMP nanoparticles was similar to that of Neoral, the current available CyA oral formulation with Cremophor EL. However, the mean residence time and the decrease of elimination constant of the particles was increased compared to Neoral. Sustained release of CyA from eroding HPMP nanoparticles was proved and as soon as the CyA molecules are released, they are absorbed by the lower segment of the small intestine.

SURFACE MODIFIED NANOPARTICLES

Introduction

As shown above, there has been a considerable effort in developing (biodegradable) constructs for the sustained delivery of therapeutic molecules. Besides drug protection, stability and ability to control drug release, targeting is also

of major importance during drug delivery. Targeting is the direct delivery of therapeutic molecules to the target cells, without damaging other tissues. Several of the above mentioned drug delivery systems accumulate in non-target tissues, as they are not target-specific. Surface properties of colloidal carrier systems play a pivotal role in their fate after intravenous injection. This is the reason why research has been focused on the development of surface modified nanoparticles to target to specific cells or organs.

Before describing the nanoscopic drug delivery systems with surface modified nanoparticles, we will first describe the types of targeting (more precisely active and passive targeting) as we feel it is important to have good insight in targeting strategies in order to understand the used techniques for development of the most appropriate targeted drug delivery systems. Afterwards we discuss some surface modified particles for active and passive targeting and to conclude we will also give some examples of particles that were modified with other aims than targeting.

Targeting

Passive targeting

In literature, “passive targeting” is used for two phenomena which are closely related, but not exactly the same. Moreover, the result of these types of targeting is opposite: in the first type “passive targeting due to size”, target cells are phagocytes and the organs of the RES. And in the second type “passive targeting due to surface modifications”, the aim is to avoid these cells.

Passive targeting due to size. After administration, small molecules and particles are removed from the blood system by renal filtration. Particles that are too large to be cleared by renal filtration, more specific molecules with a molecular weight larger than 5 kDa or even 100 kDa for condensed particles, are being removed from the blood stream by opsonization and phagocytosis[74]. Opsonization is the process in which body foreign materials are covered with opsonins, these are blood serum components that aid in the process of phagocytic recognition. As a result, the particles are recognized by macrophages and after phagocytosis, they accumulate in organs of the RES like liver, spleen, lungs and bone marrow. All the above mentioned nanoscale drug delivery systems were large enough to be taken up by phagocytosis and will end up in the RES organs. As a result, due to this first passive targeting strategy, the utility of these conventional colloidal carriers as vehicles for drug delivery to non-RES organs is inappropriate.

Passive targeting due to surface modification. A second passive targeting strategy is based on the typical vasculature of some pathological tissue. Due to the rapid growth of the tissue, the vessels for blood supply are leaky, showing a disrupted endothelial cell layer[75]. This leaky vasculature, typical for tumour tissue, inflammations and infarcted areas, offers targeting possibilities as larger particles can only penetrate in this type of tissue, leaving healthy tissue aside[76]. The fact that larger particles or molecules tend to accumulate in tumour tissue much more than

they do in normal tissues is known as the enhanced permeability and retention (EPR) effect[77] (Figure 8).

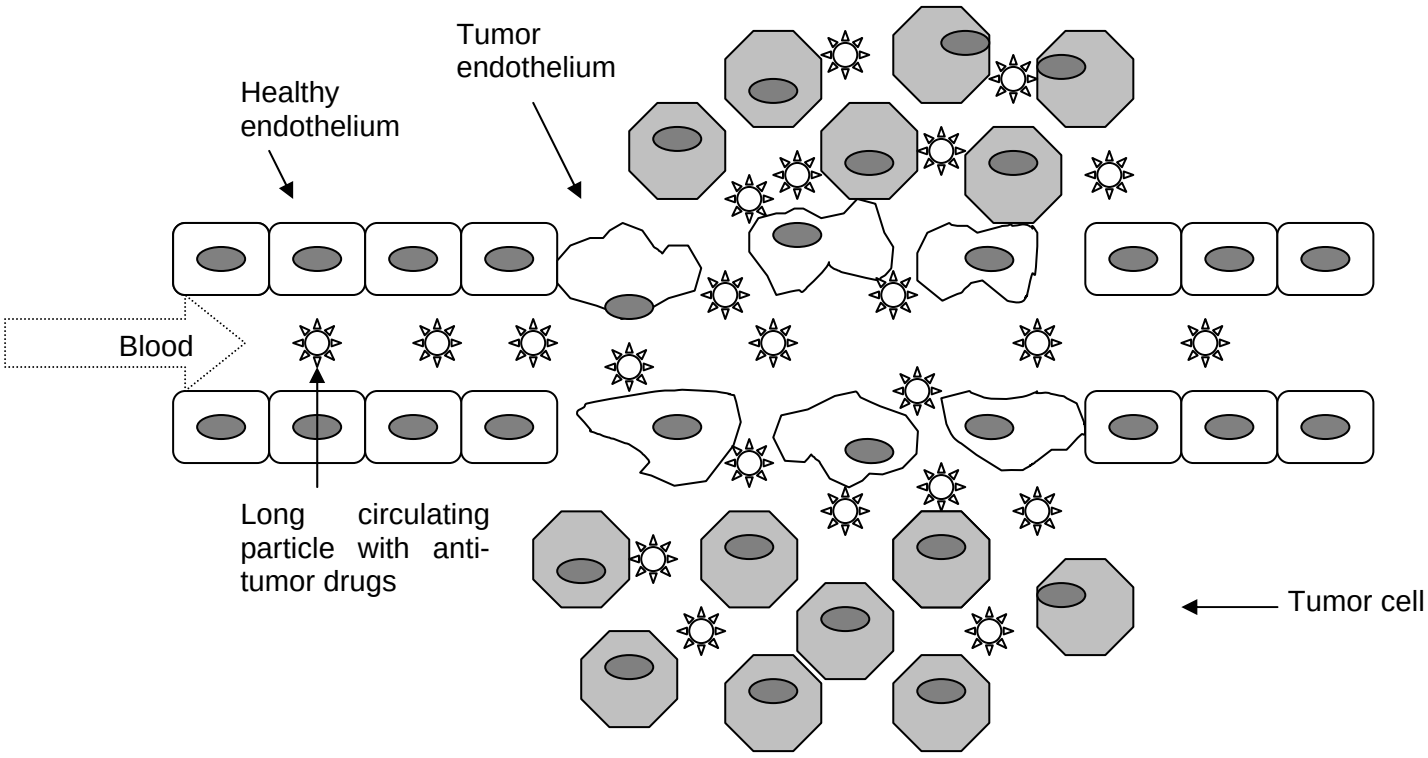


Figure 8. Schematic representation of the passive targeting strategy: long circulation particles can escape from the bloodstream to the tumour by the leaky vasculature of the tumour.

This passive targeting strategy can be achieved only by long time circulation, as conventional nanoparticles are cleared from the bloodstream within seconds by opsonization and phagocytosis[78] as described above. To avoid the binding of opsonins to the particles' surface, and as a result avoid phagocytosis, several methods of camouflaging or masking nanoparticles have been developed. Many systems make use of surface modifications that interfere with the binding of opsonin proteins. Commonly used hydrophilic polymers are poly(ethylene glycol) (PEG), poloxamines, poloxamers and polysaccharides. These coatings provide a dynamic 'cloud' of hydrophilic and neutral chains at the particle surface, which repel opsonins. PEGylation is by far the most used strategy: the particles' surface is covered with a PEG layer[79]. The presence of this layer onto the particles' surface causes not only an increase in size, but also attracts a hydrated shell around the liposome and steric hindrance is introduced[80]. Removal from the bloodstream by renal clearance, opsonization, macrophage uptake and enzymatic degradation are decreased. As a result pharmacokinetic -and hence pharmacodynamic- properties of the formulations are improved. In addition, native drug delivery systems can evoke a severe immunogenic response, especially the once containing molecules of non-human nature. PEGylated formulations often show reduced immunogenic response due to steric masking of the immunogenic sites.

Active targeting

Given the potency (and toxicity) of modern pharmacological agents, tissue selectivity is a major issue. In the delivery of chemotherapeutic agents to solid cancers this is particularly critical, since the therapeutic window for these agents is often small and the dose-response curve is steep. Drug delivery systems may function through their ability to recognize certain types of target tissue, termed as active targeting. Active targeting of a therapeutic agent is achieved by modifying the carrier system's surface by conjugation of a tissue or a cell-specific ligand.

Now we have gained insight in the necessary features to provide targeted drug delivery systems we will discuss several nanoscopic drug delivery systems described in literature that provide sustained drug delivery targeted to specific organs.

Surface modification to obtain passive targeting to non-RES organs

Doxil was the first commercially available PEGylated liposomal sustained delivery system, releasing the anti-cancer drug doxorubicin in a retarded manner within the tumor[81]. Encapsulation in liposomes containing surface-bound PEG significantly limits the distribution and elimination of doxorubicin, results in greater accumulation of the drug in Kaposi sarcoma (KS) lesions 72 hours after dosing than does standard doxorubicin, and improves drug efficacy and therapeutic index in the treatment of AIDS-KS. PEGylated liposomes containing rifampicin and isoniazid provide a drug carrier system exhibiting a sustained release in plasma and organs. The PEGylated liposomal form of these antitubercular drugs given at 1/3rd of the recommended dose was more effective than their corresponding free form, showing increased affinity to the lung tissue and uptake in RES was reduced as compared to conventional liposomes[82-84].

Perez et al.[85] tried to prepare PLGA-PEG nanoparticles loaded with pDNA for vaccination after intranasal administration. The low surface charge of PLGA-PEG nanoparticles as compared to that of PLA nanoparticles showed the presence of a PEG layer on the nanoparticles surface. They selected the appropriate conditions for the nanoencapsulation of pDNA in order to achieve a significant pDNA loading and sustained release. Encapsulation efficiencies for up to 90% could be achieved and release could be sustained for 28 days. Nevertheless, it turned out that despite the use of PVP or PVA as co-encapsulating agent, the supercoiled plasmid –the most active form of pDNA- was converted into open circular and linear forms, which are less active. This conversion to open circular and linear forms has been attributed to the sonication step in the nanoparticle formation process, even for very short periods of time (5 s).

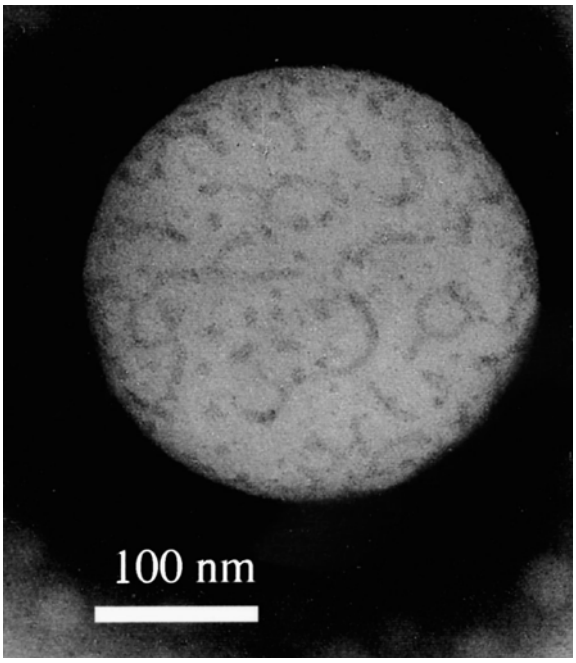


Figure 9. TEM micrograph of a plasmid DNA-loaded PLGA-PEG nanoparticle prepared by a water–oil–water solvent evaporation technique[86].

Zweers et al.[87] used PLGA-PEG for the controlled delivery of dexamethasone and rapamycin, two drugs used in the combat against restenosis after percutaneous transluminal coronary angioplasty. When PLGA-PEG particles were used, 100% of the drugs was released within 5h under physiological conditions. This was attributed to the presence of the drugs at the surface of the particles and the presence of pores in the interior of the particles. To extend the release, drug loaded nanoparticles were redispersed in an aqueous gelatin or albumin solution. As a result, sustained release could be achieved: dexamethasone for 17 days and rapamycin for 50 days. A likely explanation for the effect of protein treatment on the drug release is that the protein molecules penetrate and/or block the pores of the particles, thereby decreasing diffusion of drug through the pores. In addition, the proteins increase the viscosity of the aqueous phase in the pores resulting in a decrease of diffusion of the drug through the pores.

Very recently, Zhang et al.[88] were able to prepare poly(caprolactone-co-lactide)-b-PEG-b-poly(caprolactone-co-lactide) (PCLLA-PEG-PCLLA) nanoparticles, resulting in particles consisting of PCLLA with PEG molecules stretching out from it. 24h after injection of HCPT loaded nanogels, the HCPT concentration in plasma was almost the same as that at 8h post administration, indicating that HCPT level in blood could be maintained for an extended time. When HCPT in solution was injected, 24h post administration the HCPT concentration in the plasma could not be detected anymore. Nonetheless the presence of an outer PEG layer, the particles were accumulated in the organs of the RES, like liver and spleen, even at 24h after administration a certain level of HCPT was still maintained in all the tested RES organs. Unfortunately, the particles could not reach the tumor tissue as efficiently as the RES organs.

Zhang and Zhuo[89] used ε-caprolactone in a block copolymer with PEG to prepare nanoparticles with prolonged blood-circulation time. The poorly water-soluble anticancer drug 4'dimethyl-epipodohyllotoxin (DMEP) could be encapsulated with high efficiency in the hydrophobic PCL structure of these nanoparticles. It was shown that the release time of DMEP out of the particles was influenced by the chain length of the PEG block. When PEG with a molecular weight of 4000 g/mol was used, 90% drug was released from the polymeric nanoparticles in 50h.

Missirlis et al.[90] described the preparation of nanoparticles composed of PEG and poloxamer 407. Micellar structure are present in the bulk of these hydrogel nanoparticles and they are surrounded by a hydrophilic PEG-rich matrix. This nanoarchitecture not only allows for protection against opsonization and phagocytosis, but also sustained delivery of small hydrophobic drugs like doxorubicin can be achieved: encapsulation provided sustained release for up to one week *in vitro.* Doxorubicin was encapsulated following an *a posteriori* drug loading protocol, which avoids the exposure of the drug to the harsh conditions of radical polymerization. Nevertheless, degradation studies of doxorubicin revealed that this procedure does not fully protect the drug, most likely because of the presence of some water in the hydrophobic domains.

Owens et al.[91] have given a very good overview of studies on opsonization of polymeric nanoparticles with surface adsorbed and covalently bound PEG and PEG containing polymer layers like for example poloxamer and poloxamines.

Instead of using PEGylation to produce long circulating particles, the Couvreur group succeeded in producing a series of comb-like amphiphilic copolymers in which poly(ε-caprolactone) were covalently grafted onto a dextran backbone (Dex-PCL). These amphiphilic co-polymers have the ability to self assemble into PCL nanoparticles surrounded by a dextran layer by a technique derived from emulsion-solvent evaporation[92]. They were examined for the sustained delivery of tamoxifen, a poorly water soluble drug for oral breast cancer treatment. The PCL of the Dex-PCL nanoparticles was degraded by the *Pseudomonas* lipase. *In vitro* release studies of tamoxifen performed in phosphate buffer containing 0.5% (w/v) sodium lauryl sulfate showed a sustained release over more than 72h[93].

Surface modification to obtain active targeting

A possible way to actively target to cells is by coupling transferrin to the particles' surface. When a transferrin protein loaded with iron encounters a transferrin receptor on the surface of a cell, it binds to it and is consequently transported into the cell by endocytosis. Li et al.[94] used transferrin conjugated PEGylated polycyanoacrylate nanoparticles for the controlled delivery of pDNA to target cells. The *in vitro* release experiments showed that the cumulated pDNA release over 7 days ranged from 86% to 74.4%, depending on the concentration of the polymer in the nanogels. It was also shown *in vitro* that due to the presence of

transferrin on the nanogels' surface, the particles were directed towards target cells, as the presence of free transferrin decreased significantly the degree of cell binding of the nanogels. Besides, efforts were made to reduce the damage of pDNA during the encapsulation process by use of PVP or PVA as a stabilizer during the emulsion forming: 0.1M $NaHCO_3$ with 3% PVA (w/v) could reduce the damage of pDNA during encapsulation.

Core shell type particles with other aims than targeting

Sometimes the surface of nanoscopic particles is modified with other aims than targeting. For example, particles can be foreseen with an extra layer as an extra feature to control drug release. Most often, the core consists of a (biodegradable) polymer that can contain high amounts of drugs and that can provide a first barrier for drug release. The shell that surrounds the core can form a second barrier for (diffusional) drug release, as burst release of a certain drug might result in toxic concentrations.

Very recently Chavanpatil et al.[95] reported on Aerosol OT™ (AOT)-alginate nanoparticles for the sustained intracellular delivery of doxorubicin. The particles were formulated by emulsification-cross-linking technology and polymerization was performed by Ca^{2+}. As AOT (dioctyl sodium sulfosuccinate) has a polar sulfosuccinate head group with a large and branching hydrocarbon tail, alginate was captured inside liposome-like structures formed by AOT during the multiple emulsion/cross-linking process used. Upon cross-linking, core-shell like nanoparticles were formed with the ability to enclose small hydrophilic molecules inside the alginate core (Figure 10). Ca-alginate hydrogels get converted into soluble alginate in the presence of sodium ions. As a result, *in vitro* release studies in PBS demonstrated near zero order drug release over a 15 day period. Drug release was even slower in cell culture medium, probably due to the presence of divalent metal salts slowing down the sodium-calcium exchange. Doxorubicin in nanoparticles demonstrated significantly higher cytotoxicity against two breast cancer cell lines when compared to doxorubicin in solution.

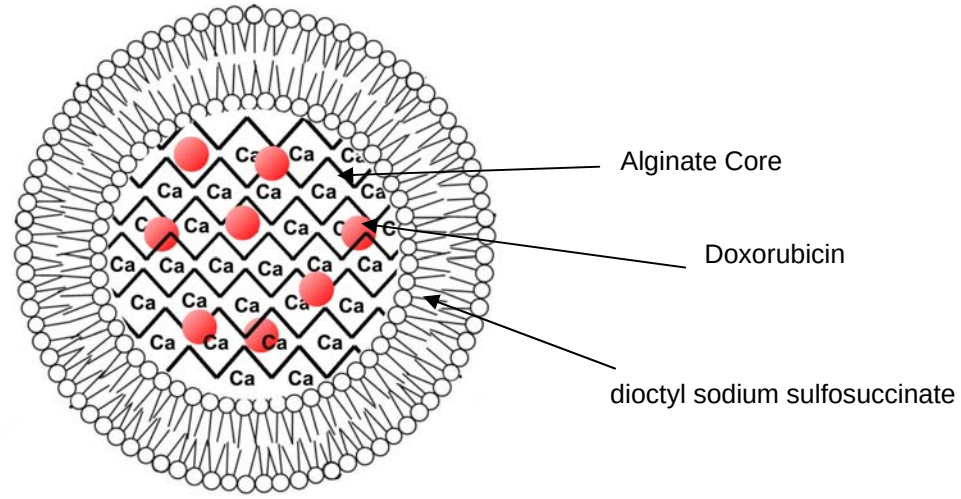


Figure 10. Proposed structure of AOT-alginate nanoparticles. The inner core consist of alginate cross-linked with calcium, surrounded by one or more bilayers of AOT. Figure not drawn to scale.

Hitzman et al.[96-98] presented an elaborated study on lipid coated nanoparticles prepared by spray drying. The particles' core was composed of poly(glutamic acid), poly(lysine) and lactose. Shell materials consisted of various combinations of tripalmitin, tristearin, cetyl alcohol and stearyl alcohol. The particles were prepared to be used as a respirable sustained delivery system to treat lung cancer with 5-fluoroacil (5-FU). Depending on the composition of the shell and especially the core, *in vitro* release durations in excess of 24h could be obtained from these lipid coated nanoparticles. Particles without a lipid shell and composed of 5-FU with polymeric cores excipients are observed to undergo relatively rapid dissolution and release of 5-FU, suggesting that the lipid shell is the rate limiting step for the release of 5-FU. The nanoparticles were successfully aerosolized using an ultrasonic nebulization system. *In vivo* assays of the release characteristics were carried out in hamsters following intratracheal instillation and aerosol inhalation. By the two methods, the lipid coated nanoparticles effectively reached the lower respiratory tract and similar release profiles *in vivo* and *in vitro* were observed.

Another possibility is that the surrounding shell provides a responsive reaction in order to make particles that interact with environmental changes: it can react to light, pH, enzymes or temperature. Jeong et al.[99] reported on core-shell type nanoparticles composed of PLGA-grafted dextran for use as a colon drug carrier. PLGA formed the inner-core and acted as a incorporating domain for doxorubicin and dextran formed the hydrated outer shell, able to degrade in the colon as dextranase is presented there. But in our opinion, this system is less suitable for colon-targeting, as sustained release was observed both in the absence as the presence of dextranase, although the drug release became faster in the presence of the enzyme. As a result, most of the drugs will be released before reaching the colon.

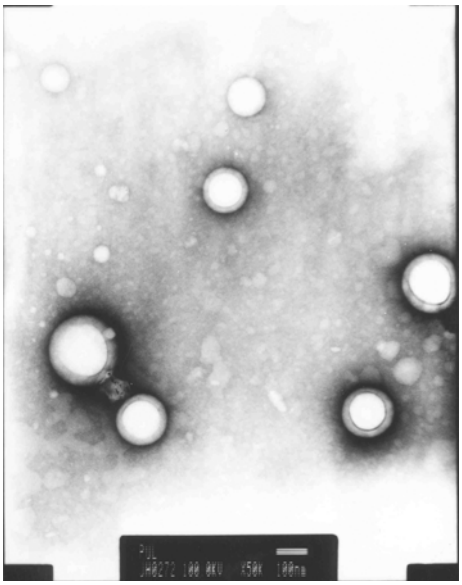


Figure 11. TEM photographs of core-shell type nanoparticles composed of PLGA-grafted dextran[100]

Recently, our group proposed dextran and PEG nanoparticles provided with a lipid coating for the sustained delivery of therapeutic molecules. The particles were obtained by using liposomes as a nanoscopic reactor: the liposomes were filled with a polymerizable dextran or PEG solution and these polymers were subsequently cross-linked by means of UV in the interior of the liposomes. The cores of the particles were composed of hydroxyethylmethacrylated dextran or PEG (respectively dex-HEMA and PEG-HEMA) which are degradable under physiological conditions. The degradation time of these particles could range from some hours to several weeks. Moreover, the particles could be uploaded with some model proteins and sustained release could be achieved.

The use of this preparation technique offers several advantages over other well established techniques like for example emulsion polymerization or controlled precipitation. (1) The size of the nanogels can be controlled by using membranes with the appropriate pore size in the preparation of the liposomes. (2) organic solvents are not used to avoid drug inactivation. (3) We also showed that it was possible to use PEGylated and pH sensitive lipid coatings in order to provide long circulating nanoparticles that can be taken up by cells and loose their protecting lipid coating in the acidic environment of the endosomes.

CONCLUDING REMARKS

This literature overview shows that, especially within the last decade, different concepts and systems have been developed for sustained drug delivery from nanoscopic particles. By use of nanoscopic materials, different targets can be reached: specific cells, like tumor cells or phagocytes, but also specific organs, like the RES organs (liver, spleen, bone marrow), the lungs, the gastrointestinal tract or even the eye. The altered body biodistribution combined with a prolonged retention

time of the therapeutic molecules in various tissues and protection against many possible “attacks” in the human body may be responsible for the better therapeutic effect of drug loaded nanogels.

Nevertheless, a long way of research is still open for exploration: the complex processes of gradual nanosphere degradation and erosion within the vasculature and the kinetics of drug release into the blood have been poorly addressed. Not only the clearance kinetics of the nanoparticle from the blood should be considered, but also the *in vivo* process of gradual nanosphere matrix degradation and the extent of drug release must be worked out in relation to a pharmacologically desired free drug profile. Other critical considerations are the biocompatibility of the pharmaceutical ingredients, the shelf life and the ease and cost of large scale manufacturing. Especially the latter two are important considerations which are often ignored in the first steps of scientific research.

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ON THE SYNTHESIS AND CHARACTERIZATION OF BIODEGRADABLE DEXTRAN NANOGELS WITH TUNABLE DEGRADATION PROPERTIES

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Abstract

Hydrogels are widely investigated as carriers in drug delivery. To be suitable carriers for intracellular drug delivery the hydrogels should be small enough to be able to enter cells. This second chapter reports on the synthesis and characterization of both lipid-coated and 'naked' biodegradable hydroxyethyl methacrylated dextran (dex-HEMA) nanogels. Dynamic Light Scattering (DLS), atomic force microscopy and transmission electron microscopy showed that lipid-coated nanogels could be obtained by polymerization of an aqueous dex-HEMA solution entrapped in SOPC:DOTAP liposomes (SOPC and DOTAP respectively being 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and 1,2-dioleoyl-3-trimethylammonium propane chloride). Naked dex-HEMA nanogels were prepared by removing the lipid coating by Triton X 100. DLS measurements on dex-HEMA nanogels stored in buffer at 37°C revealed that the degradation time depended on the cross-link density of the nanogels: dex-HEMA nanogel prepared from dextran lowly substituted with HEMA degraded fast while it took days to weeks for nanogels prepared from highly substituted dextran. Furthermore, confocal laser scanning microscopy showed that SOPC:DOTAP coated dex-HEMA nanogels can be taken up by VERO cells.

INTRODUCTION

Many molecules which are under development as therapeutic agents have targets located intracellularly. Examples are certain proteins, oligonucleotides, plasmid DNA and interference RNA. However, most of these molecules show a poor cellular uptake and become easily degraded in extra- and intracellular media. Appropriate nanosized delivery systems which protect and deliver the drug molecules intracellularly are therefore required[1]. While intracellular drug release has been shown to occur from many types of nanoparticles, it remains however rather unknown which parameters govern the drug release process once the nanoparticles enter the cell. This is partly due to difficulties in characterizing quantitatively the biophysical behavior of nanoparticulate matter in living cells. As a consequence it remains also very challenging to design drug loaded nanoparticles with optimized intracellular release properties. Indeed, while micron sized particles (which remain extracellularly after injection) with tunable release properties have been well studied[2-8], however, little attention has been paid so far to nanoparticles which could slowly release the drug in the cell after uptake by the cells.

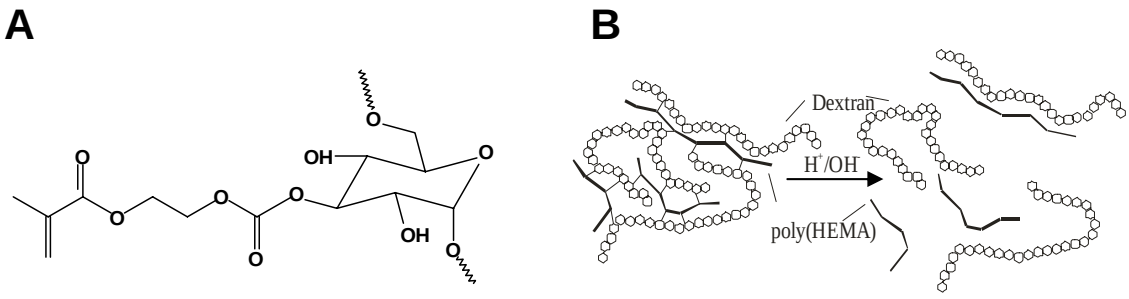

Figure 1. (A) Chemical structure of the monomer in dex-HEMA, i.e. glucopyranose substituted with HEMA. (B) Schematic representation of the dex-HEMA polymer network before (left) and after (right) degradation. The HEMA cross-links degrade through hydrolysis of the carbonate esters, resulting in dextran chains and poly(HEMA)

In previous work the physicochemical and release properties of biodegradable hydroxyethyl methacrylate dextran (dex-HEMA, Figure 1A) hydrogels and microgels was investigated[3,9-13]. Dex-HEMA gels are biocompatible[14] and degrade spontaneously under physiological conditions[12]. Drug molecules can be entrapped in a dex-HEMA network and controlled degradation of the network can result in sustained drug release during several days. The degradation rate of dex-HEMA hydrogels and dex-HEMA microgels is dependent on their cross-link density, which can be varied by altering the degree of substitution of the dextran (DS, i.e. the numbers of HEMA groups per 100 glucose units) and the dex-HEMA concentration. Recently, we became interested in dex-HEMA *nanogels* for sustained intracellular drug delivery. This chapter deals with the first

step in this research: it describes the preparation of dex-HEMA nanogels and tuning of their degradation rate by changing their composition.

Several methods have been reported to obtain nanoscopic hydrogel particles. A commonly used method is emulsion polymerization[15-17]: a stable emulsion is formed and next the droplets of the aqueous polymer solution are polymerized to form the nanogels while the surrounding organic solvent is removed by evaporation[15] or extraction and dialysis[17]. Major drawbacks of this method are the use of organic solvents and the need of energy (e.g. sonication) to form the emulsion, which may inactivate the entrapped therapeutic molecules. Also, nanogels obtained by emulsion polymerization are often strongly polydisperse in size. An interesting approach, without the use of organic solvents, has been recently described by the Levon group[18,19]: nanogels were made by UV polymerization of a solution of acrylamide and N, N'-methylenebis(acrylamide) entrapped in liposomes. After the formation of these nanogels, the surrounding lipid layer was removed by adding a detergent. Besides the absence of organic solvents, another advantage of this method is that the size and polydispersity of the nanogels can be easily controlled since the liposomes, which act as a container in which the nanogels are composed, are formed by multiple extrusion through a membrane with a well defined pore size. Another benefit of this method is that both non-coated (naked) nanogels and lipid-coated nanogels can be made, which may be attractive for certain drug delivery applications. Recently, Patton et al.[20] also made use of liposomes as nanoreactors for making artificial oxygen carriers as blood substitutes[20]: bovine hemoglobin was entrapped in poly(acrylamide)/poly(*N*-isopropylamide) nanogels prepared in liposomes. Another recent study reported on actin nanogels prepared by polymerizing actin monomers to filamentous structures in phosphatidyl choline based liposomes[21,22]. These lipid-coated actin nanogels were aimed at revealing how the cytoskeleton, coupled to the surrounding cell membrane, determines the cell shape during dynamic processes.

The aim of this chapter is threefold. First, we wonder whether both lipid-coated and naked dex-HEMA nanogels could be obtained by using liposomes as a nanoreactor. Second, we investigate whether in this way dex-HEMA nanogels could be obtained which differ in degradation properties. Third, we are interested to know whether the obtained dex-HEMA nanogels could be taken up by cells.

EXPERIMENTAL SECTION

Dex-HEMA preparation and characterization

Dex-HEMA batches were prepared and characterized according to a method described elsewhere[20]. Dextran (Fluka, from *Leuconostoc ssp.*) with a molecular weight M_n of 19 000 g/mol was used. The degree of substitution (DS, the number of HEMA groups per 100 glucopyranose residues of dextran) was determined by proton nuclear magnetic resonance spectroscopy (H-NMR) in D_2O with a Gemini 300

spectrometer (Varian). The DS of the samples used in the present study were 2.9, 5.0, 7.5 and 18 respectively.

Preparation of dex-HEMA nanogels

As outlined in the introduction, dex-HEMA nanogels were prepared using liposomes as a nanoscaled reactor. The procedure is schematically depicted in Figure 2.

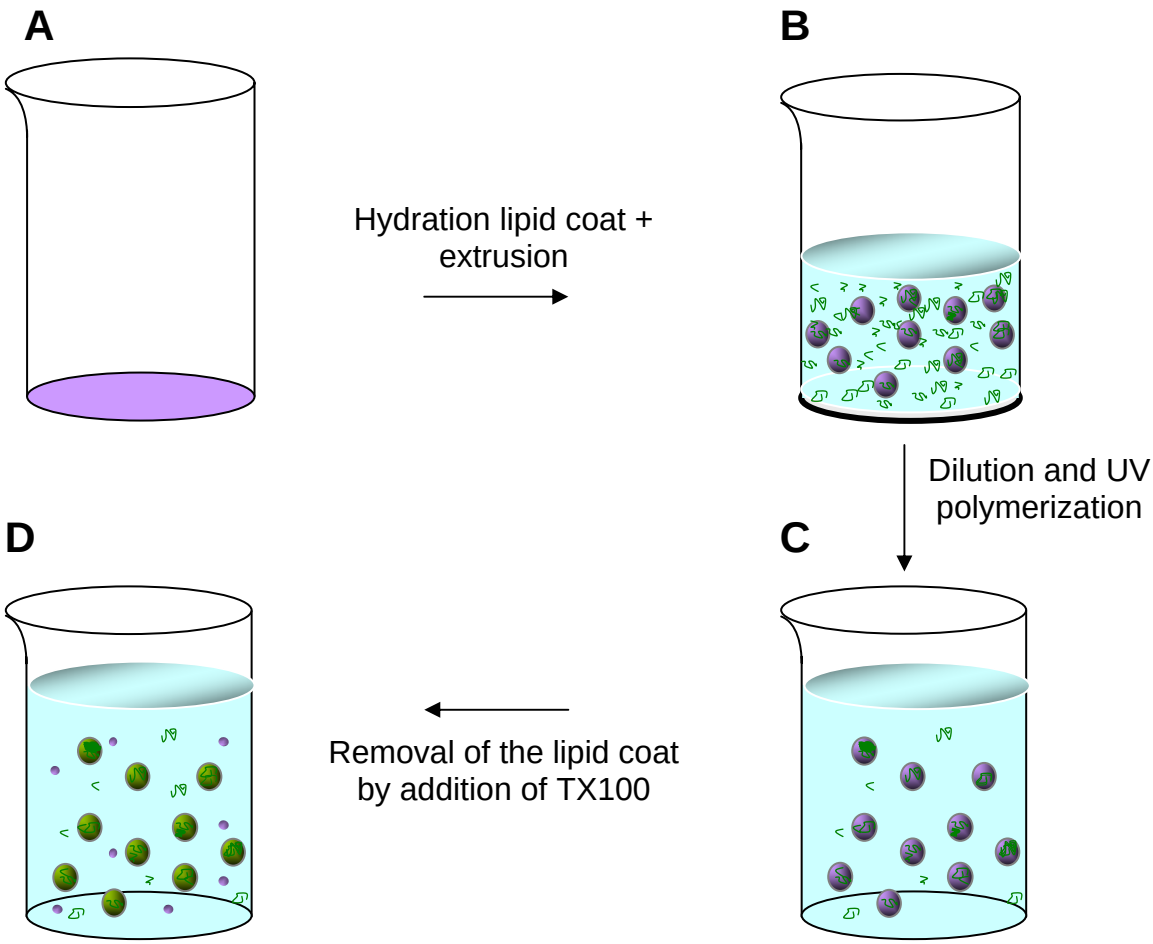

Figure 2. Schematic representation of lipid coated and naked nanogels formation

A conventional procedure to prepare the liposomes was used. A 5 mg lipid film of SOPC:DOTAP (9:1) (SOPC and DOTAP respectively being 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine and 1,2-dioleoyl-3-trimethylammonium propane chloride, Avanti Polar Lipids) was made by dissolving the lipids in chloroform and drying this solution under a nitrogen flow while gently spinning the vial. This resulted in a thin lipid film on the bottom of the vial (Figure 2A). The vial was placed under vacuum for at least 4 hours to remove all remaining chloroform. Subsequently, this dry lipid film was hydrated with 1 mL of a dex-HEMA solution (i.e. 20% (w/w) dex-HEMA in 50 mM phosphate buffer (PB) at pH 7.0) which contained 0.05% (w/w) Irgacure 2959 (Ciba Specialty Chemicals) as a photo

initiator. The resulting dispersion was placed at 25°C for 30 minutes while vortexing every 5 minutes. This dispersion of large vesicles was aged overnight. Next, the dispersion was extruded with a hand-held syringe fitted with a standard filter holder (Avanti Polar Lipids) provided with a 450 nm polycarbonate membrane (Schleicher & Schuell) (Figure 2B). After 11 back-and-forth passages of the dispersion through the extrusion membrane, the liposome dispersion was diluted, 10 times with PB. This dilution was necessary as not all the dex-HEMA was entrapped in the liposomes. If not diluted this "free" dex-HEMA would form a gel in the polymerization step, thereby enclosing the liposomes in a polymer matrix. The dispersion was subsequently exposed to UV light (365 nm from a Bluepoint 2.1 UV source, Honle UV Technology) at 25°C for 450 s which cross-linked the dex-HEMA solution in the liposomes with the formation of "lipid-coated dex-HEMA nanogels" (Figure 2C). To obtain "naked dex-HEMA nanogels" the lipid layer was removed by adding 20 µL of a 100 mM solution of the detergent Triton X 100 (TX 100, Merck) to 1 mL of the liposome dispersion (Figure 2D).

Dynamic light scattering (DLS) analysis on dex-HEMA nanogels

Dynamic light scattering measurements were done with a Malvern Autosizer 4700 at a fixed angle of 90°. A He-Ne laser (633 nm, 25 mW) was used as a light source. PB, used to dilute the dispersions of the nanogels for DLS measurements, was filtered through a 0.1 µm Millipore Durapore® filter. Polystyrene nanospheres (220 ± 6 nm; Duke Scientific Corporation) were used to check the performance of the DLS instrument. The mean hydrodynamic diameter of the particles (d_h) was computed from the intensity-intensity correlation function using the Malvern software package based on the theory of Brownian motion and the Stokes-Einstein equation

$$D = \frac{kT}{3\pi\eta d_h} \qquad \text{eq. 1}$$

where D is the diffusion coefficient (the primary parameter obtained from DLS measurements), k the Boltzmann constant, T the temperature (298 K) and η the viscosity of the solvent (0.96 cP).

To study the degradation of the nanogels in function of (degradation) time, a cuvette was filled with 1.2 mL of the nanogel dispersions and sealed with Parafilm™ to avoid contamination with dust particles. The cuvette was placed at 37°C and DLS measurements on the degrading nanogels were performed at regular times.

Atomic force microscopy (AFM) on dex-HEMA nanogels

Atomic force microscopy images of the nanogels were recorded in air at ambient temperature with an Autoprobe CP (Thermomicroscopes) using a 5 µm

scanner. The images were recorded in the intermittent-contact (tapping) mode. Pointprobe "Low Frequency Non-Contact" Sensors (Nanosensors) were used. These cantilevers have a resonance frequency around 150-170 kHz, a typical spring constant of about 50 N/m and an integrated Si tip with a radius of curvature at the apex around 10 nm. To minimize particle deformation due to tip indentation, all the images were acquired in the so-called "soft-tapping" conditions with a ratio between the set-point amplitude and the free amplitude of vibration larger than 0.9.

The nanogels made for AFM measurements were prepared from dex-HEMA and photo initiator in destilled water (instead of PB). After polymerization, the free dex-HEMA, initiator and mixed micelles in the water surrounding the lipid-coated dex-HEMA nanogels were removed by dialysis using SpectraPore 50 kDa membrane tubes (dialysis during one week, against destilled water, changing the water once a day) at 4°C (to avoid degradation of the dex-HEMA nanogels[12]). Using water to dissolve the dex-HEMA (in stead of PB) and intensive dialysis of the nanogel dispersion was necessary to avoid the deposition of salts and micelles on the mica surface, which would complicate the AFM imaging of the nanogel.

Negative stain transmission electron microscopy (TEM) on dex-HEMA nanogels

Lipid-coated and naked dex-HEMA nanogels were adsorbed on glow discharged Formvar-carbon-coated copper grids, subsequently washed twice in hepes buffered saline solution and negatively stained with 2% (w/v) uranyl acetate. The ultrastructure was analyzed with a Tecnai 10 electron microscope (FEI Company, Eindhoven, The Netherlands) at 100 kV acceleration voltage.

Cellular uptake of dex-HEMA nanogels

African green monkey kidney (VERO) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 2 mM glutamine, 10% heat-inactivated fetal bovine serum and 1% penicilline-streptomycine. Cells were prophylactically treated against mycoplasma with Plasmocin (Invivogen). The cells were seeded onto sterile glassbottomed culture disks (MatTek Corporation), allowed to grow and to adhere for 1 day (at 37°C in a humidified atmosphere containing 5% CO_2) before applying the nanogel dispersions.

For evaluation of the cellular uptake of the dex-HEMA nanogels, the nanogels were fluorescently labeled with Texas Red labeled dextran (70 kDa, Molecular Probes). Therefore 60 µL of a Texas Red dextran solution (25 mg/mL) was added to the 1 mL dex-HEMA/photo initiator solution used to hydrate the lipid film. To remove the Texas Red dextran which was not entrapped in the liposomes we used Microcon™ centrifugal filter devices (molecular weight cut off 100 kDa, Millipore Corporation). After centrifugation (at 14000×g for 24 minutes), the fluorescent dex-HEMA filled nanogels were recovered by inverted spin and diluted

ten times with DMEM before UV polymerization. DLS measurements showed no remarkable change in size after centrifugational filtration (data not shown).

200 µL of lipid-coated nanogels (containing Texas Red labeled dextran) were added to the cells in one well and incubated for 1 h at 37°C. The cells were washed three times with PB before imaging by confocal laser scanning microscopy (CLSM, Bio-Rad MRC 1024) using a 60 × water immersion objective and a krypton/argon laser (568 nm) for the excitation of the Texas Red labeled nanogels.

RESULTS AND DISCUSSION

Loading liposomes with dex-HEMA

The first step in the preparation of dex-HEMA nanogels was the loading of the liposomes with dex-HEMA. It is well known that liposomes can be formed upon hydration of a thin lipid film: the hydrated lipid film detaches during agitation and self-closes to form large multilamellar liposomes[23]. Figure 3 shows DLS measurements on the dispersions obtained by hydrating a SOPC:DOTAP lipid film with a dex-HEMA/photo initiator solution respectively before and after extruding the dispersion through a 450 nm polycarbonate membrane. It is clear that before extrusion strongly polydisperse, micron sized, badly defined aggregates exist. Upon extrusion through the polycarbonate membrane, the aggregates turn into (less polydisperse) vesicles of about 400 nm in diameter, approximately being the pore size of the used polycarbonate membrane.

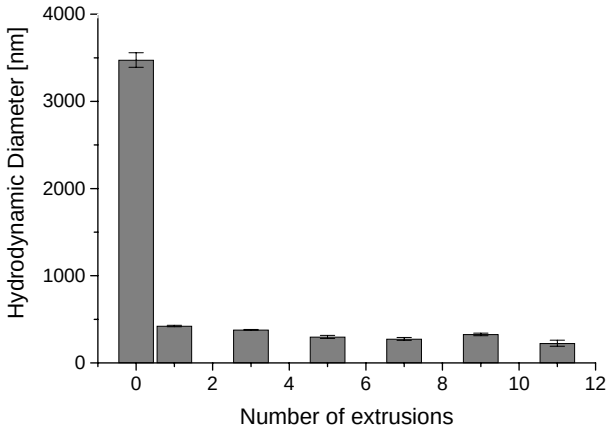

Figure 3. DLS measurements on liposomes (SOPC:DOTAP (9:1)) loaded with dex-HEMA before polymerization by UV light. The lipid film was hydrated with a 20% (w/w) dex-HEMA (DS 5.4) solution. The x-axis indicates how many times the liposome dispersion was extruded through the polycarbonate membrane. The polydispersity of the particles before extrusion and after one extrusion was >0.7. After that polydispersity did never exceed 0.3.

Overall the DLS data in Figure 3 show that liposomes are indeed formed after hydrating the SOPC:DOTAP film with (the rather viscous) dex-HEMA/photo initiator solution followed by extrusion through a 450 nm membrane. Extrusion of the liposome dispersion through 100 nm membranes was not feasible, probably due to the high viscosity of the dispersion.

We also tried to reduce the size of the liposomes obtained after hydrating the lipid film with dex-HEMA/photo initiator solution by sonication (using a tipsonicator), according to the method described by Kazakov et al.[18]. However, small metal particles were coming of the tip sonicator and interfered strongly with the DLS measurements which made it impossible to accurately characterize the obtained liposomes by DLS.

Evidence for dex-HEMA nanogels from DLS measurements

In the next step, the liposomes, as characterized in Figure 3, were exposed to UV light to polymerize the dex-HEMA in the liposomes. Figure 4A shows that the average size of the nanoparticles did not change upon polymerization.

Subsequently TX 100 was added to the dispersion to solubilize the lipid coating. Particles of about 400 nm in size could still be detected (Figure 4A), a first evidence that polymerization of the dex-HEMA in the liposomes occurred. However, also much smaller particles (~12 nm) were detected (Figure 5). These were probably mixed micelles formed by TX 100, SOPC and DOTAP. They indicated that TX 100 was able to remove the lipid layer from the particles resulting in a mixture of “naked” dex-HEMA nanogels and micelles.

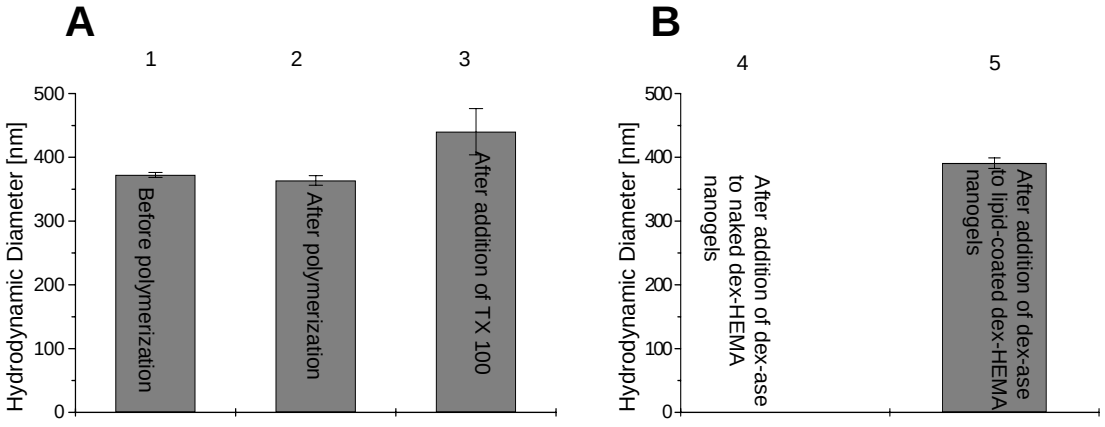

Figure 4. (A) Bar 1 shows the hydrodynamic diameter of the dex-HEMA containing liposomes described in Figure 3 before UV polymerization. Bar 2 represents the hydrodynamic diameter of the corresponding particles after UV treatment. Bar 3 shows the hydrodynamic diameter of the particles after addition of TX 100. (B) Bar 4: DLS on naked dex-HEMA nanogels treated with dextranase revealed that the nanogels were not present anymore. Bar 5 shows the DLS data obtained after adding dextranase to lipid-coated dex-HEMA particles.

Another indication for the removal of the lipid layer was the significant drop of the intensity of the light scattered by the dispersion after adding TX 100 (data not shown). The removal of the lipid layer lowers the difference in refractive index between the nanogels and the dispersion solvent, which explains the lower intensity of the scattered light.

Although the DLS measurements described above indicated the formation of naked dex-HEMA nanogels we tried to confirm that the 400 nm particles in Figure 4A and Figure 5 were indeed naked dex-HEMA nanogels. Therefore 20 µL of a dextranase solution (10U/mL) was added to 1 mL of a dex-HEMA nanogel dispersion. Figure 4B shows that no nanoparticles were detected anymore after addition of dextranase. Clearly, this was attributed to the degradation of the nanogels. However, when dextranase was added to the (still) lipid-coated dex-HEMA nanogels, nanoparticles remained present (Figure 4B). This was explained by the fact that the enzyme could not pass through the lipid coating. These observations all together indicate that naked dex-HEMA nanogels were indeed obtained after UV polymerization of dex-HEMA filled liposomes followed by TX 100 treatment.

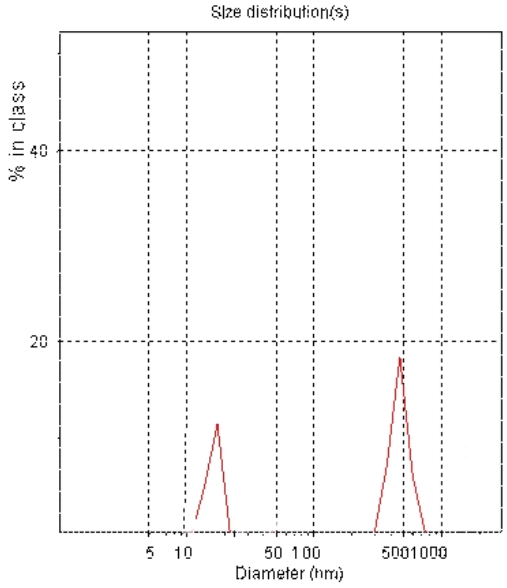

Figure 5. Typical outcome of a DLS experiment on lipid-coated dex-HEMA nanogels treated with TX 100. The x-axis shows the hydrodynamic diameter of the observed particles. The SOPC:DOTAP (9:1) lipid film was hydrated with a 20% (w/w) dex-HEMA (DS 5.4) solution. The peak with an average size of 12 nm is attributed to micelles, the one with an average size of 450 nm comes from naked dex-HEMA nanogels.

Microscopic characterization of the dex-HEMA nanogels

Confocal (fluorescence) microscopy is limited in imaging of particles of a few hundred nanometers. Therefore we preferred to further characterize the nanogels

by AFM and negative stain EM. Figure 6A shows an AFM image of SOPC:DOTAP coated dex-HEMA nanogels: the lipid coating is clearly visible at the surface of the dex-HEMA nanogels as a rough ring around the particle. The size of the lipid-coated nanogels is some hundreds of nanometers, which is in well agreement with the size data obtained by DLS, although little bit smaller. This is maybe due to the drying of the particles, which is necessary for AFM analysis. Figure 6B is an AFM image of nanogels treated with TX 100: compared to Figure 6A a more smooth line confines the nanoparticles suggesting that the lipid coating has indeed been removed.

EM on the nanogels further confirmed the AFM and DLS data: the surface of the lipid-coated dex-HEMA nanogel seems different from that obtained after addition of TX 100. The lipid surface of the coated nanogels was clearly visible on EM images (Figure 7A) while it disappeared upon treating the lipid-coated nanogels with TX 100 (Figure 7B).

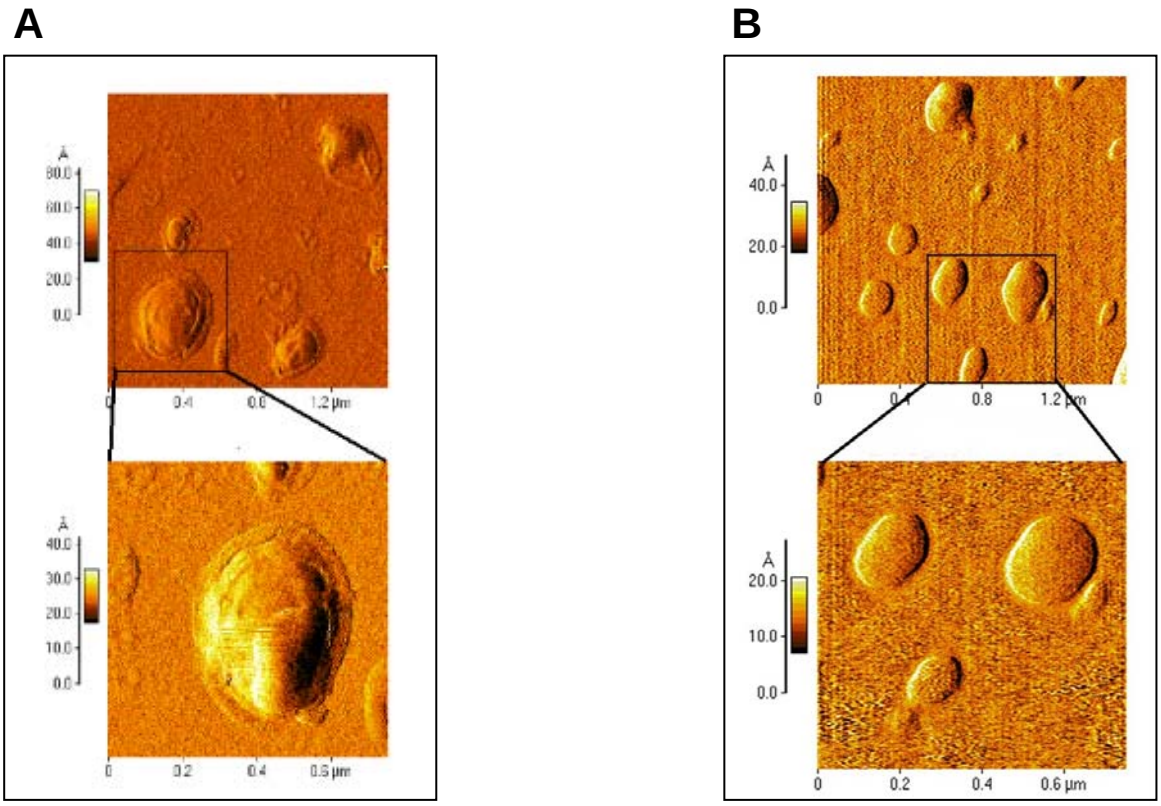

Figure 6. (A) AFM image of SOPC:DOTAP coated dex-HEMA nanogels. The SOPC:DOTAP (9:1) lipid film was hydrated with a 20% (w/w) dex-HEMA (DS 18) solution. (B) AFM image of naked dex-HEMA nanogels obtained by adding TX 100 to the dispersion imaged in (A).

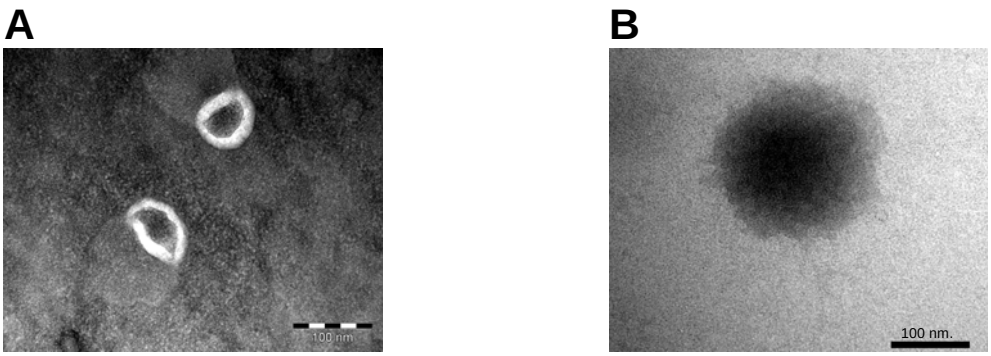

Figure 7. (A) Negative stain EM image of SOPC:DOTAP coated dex-HEMA nanogels. The SOPC:DOTAP (9:1) lipid film was hydrated with a 20% (w/w) dex-HEMA (DS 18) solution. (B) Negative stain EM image of naked dex-HEMA nanogels obtained by adding TX 100 to the dispersion imaged in (A).

Monitoring the degradation of dex-HEMA nanogels

As schematically shown in Figure 1B dex-HEMA hydrogels consist of dextran chains which are mutually cross-linked by hydroxyethyl methacrylates. Our group, as well as the Hennink group, have well documented[3,12,24,25] that the HEMA cross-links in dex-HEMA hydrogels spontaneously degrade by hydrolysis of the carbonate ester links formed between the hydroxyethyl methacrylate groups and the dextran chains (Figure 1A). Especially the cross-link density (determined by both the dex-HEMA concentration in the gel and the DS of the dex-HEMA) determines the degradation rate of dex-HEMA hydrogels. It was shown experimentally that, the higher the cross-link density, the longer it takes for dex-HEMA gel slabs[3,12,25] and dex-HEMA microgels[3,24] to degrade. We wondered whether also dex-HEMA *nanogels* could be obtained which show different degradation times. Tunable degradation properties could be of interest to regulate the drug release from the nanogels after uptake by cells.

Lipid-coated and naked dex-HEMA nanogels were dispersed in PB (pH 7.0) at 37°C and their size was measured by DLS at different times. The behavior of lipid-coated nanogels in PB is depicted in Figure 8 while Figure 9 shows the behavior of the naked nanogels. Figure 8A shows that the size of “empty” SOPC:DOTAP liposomes (i.e. without dex-HEMA) in PB (at 37°C) does not change drastically in 2 weeks time. Figure 8B, C and D reveal that nanoparticles remain to exist for at least 2 weeks when lipid-coated dex-HEMA nanogels are dispersed at 37°C in PB (independent on the DS of the dex-HEMA).

At a first sight one could conclude that the dex-HEMA nanogels in the liposomes do not degrade. However, another explanation could be that hydrolysis of the dex-HEMA nanogels in the liposomes does occur but that the degradation products of the nanogels (being dextran chains and poly(HEMA)[12], Figure 1B) remain in the liposomes as these are stable for weeks (see Figure 8A). The latter hypothesis was confirmed by the following experiments: lipid-coated nanogels (DS 2.5; 5.4 and 8.9) were allowed to degrade for respectively 5, 14 and 18 days. After

that period TX 100 was added. In this way only particles of about 12 nm were still detected. This proved that the nanogels in the liposomes were indeed completely degraded into a dextran and poly(HEMA) solution.

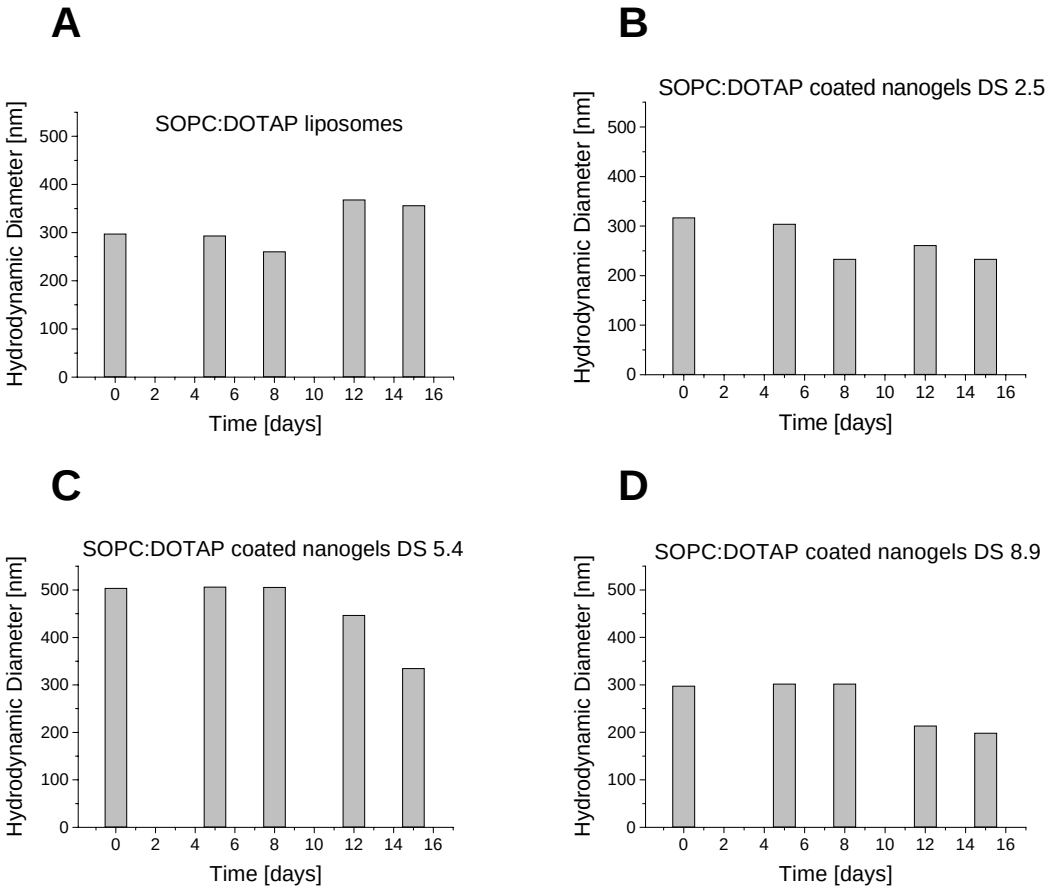

Figure 8. DLS measurements on SOPC:DOTAP (9:1) liposomes (A) and SOPC:DOTAP (9:1) coated nanogels (B,C,D) stored in PB at 37°C. To prepare the dex-HEMA nanogels the SOPC:DOTAP (9:1) lipid film was hydrated with a 20% (w/w) dex-HEMA solution. The DS of the dex-HEMA was respectively 2.5 (B), 5.4 (C) and 8.9 (D). At this moment it is not clear why the DS 5.4 nanogels were larger than the DS 2.5 and 8.9 nanogels. Data shown are the result of one set of experiments. Repeated experiments revealed the same results.

Theoretically one could argue that the turnover from the gels into the solution of degradation products would increase the osmotic pressure inside the liposomes which should rupture this lipid layer, especially as the lipid membranes are not permeable for the large dextran chains (19000 g/mol) used to synthesize the dex-HEMA in this study. Indeed, we showed previously that the osmotic pressure of a dex-HEMA gel increases upon degradation, which also explains the swelling of degrading dex-HEMA gels[25,26]. However, the following considerations point out that the increase in osmotic pressure is probably too low to osmotically destroy the liposomes. Following the method of Mui et al.[27] the rupture strength (τ) of a SOPC:DOTAP (9:1) film was determined and turned out to be 0.075 N/m. From Laplace's law (eq. 1)

$$\tau = \frac{\Delta P \times r}{2} \qquad \text{eq. 2}$$

in which ΔP (in N/m²) stands for the pressure difference over the lipid membrane, τ (in N/m) being the tensile strength and r (in m) the radius of the liposome, one can calculate that for a 450 nm SOPC:DOTAP (9:1) liposome the pressure increase needed to overcome the tensile strength, i.e. to rupture the membrane, should exceed 666 kPa. We measured previously the osmotic pressure of completely degraded dex-HEMA hydrogels[25,26] and could estimate that the osmotic pressure of the solutions obtained by degradation of the dex-HEMA nanogels used in Figure 8B, C and D (assuming the dex-HEMA concentration of these gels is 20% (w/w)) is only 120 kPa and thus insufficient to rupture the liposomal membrane. This supports the idea that the SOPC:DOTAP lipid-coated dex-HEMA nanogels do degrade yielding SOPC:DOTAP liposomes filled with a dextran/poly(HEMA) solution and that the degradation products remain in the liposomal vesicles.

Figure 9 (B, C and D) shows DLS data obtained on naked dex-HEMA nanogels stored in PB at 37°C. As Figure 5 already illustrated, DLS measurements on naked dex-HEMA nanogels indicated the presences of both small micelles and larger nanogels. The y-axis in Figure 9 plots the average size of the nanogels. Clearly, the average size of the nanogels in the dispersions decreases (rather slowly) in time, indicating that they indeed degrade. Especially, as seen earlier for dex-HEMA hydrogel slabs and dex-HEMA microgels[3,12,24,25], the degree of cross-linking influences the degradation rate of the dex-HEMA nanogels: the higher the DS of the dex-HEMA (i.e. the higher the cross-link density of the dex-HEMA nanogels) the longer nanogels can be detected in the dispersions (compare Figure 9B, C and D). Note that before degradation starts (i.e. at t=0 in Figure 9) dex-HEMA nanogels DS 2.5 are smaller than the nanogels DS 5.4 and 8.9. This is possibly due to the fact that the dex-HEMA nanogels DS 2.5 were already partially degraded at the time of the first DLS measurement (as dex-HEMA nanogels DS 2.5 degrade very fast due to the low cross-link density).

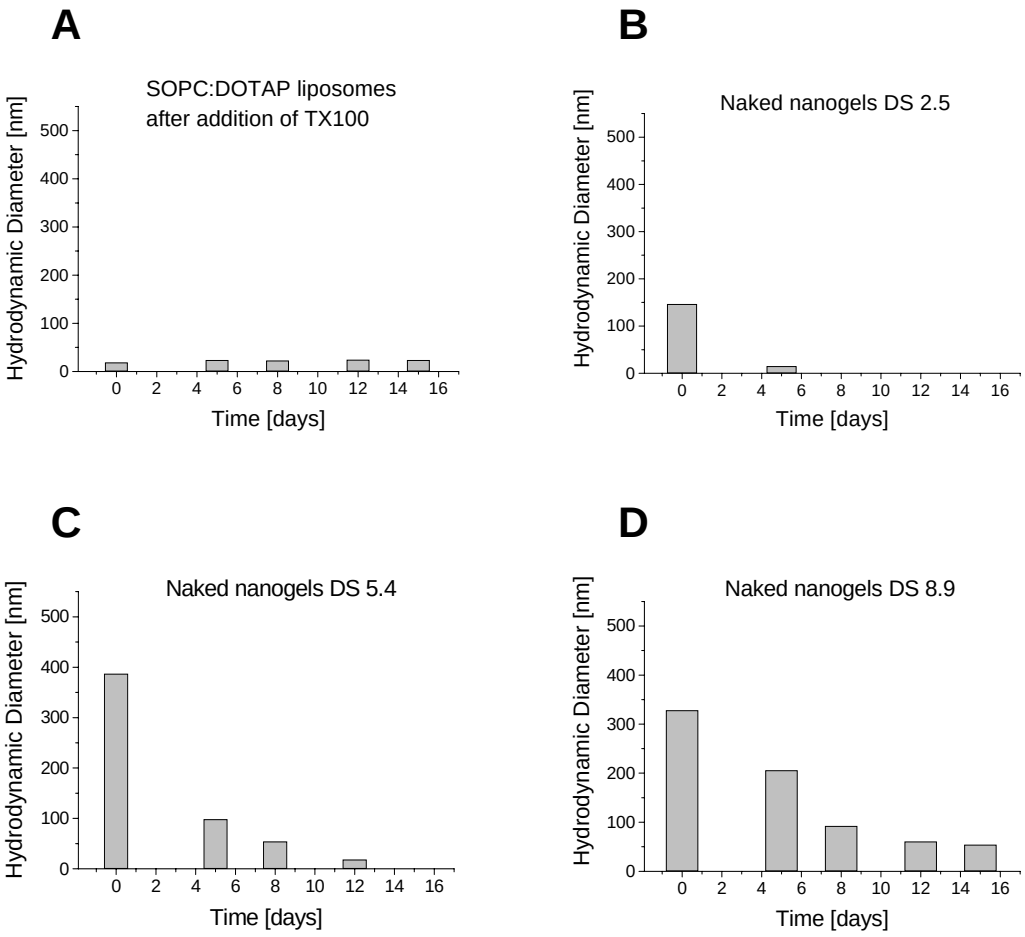

Figure 9. DLS measurements on SOPC:DOTAP (9:1) liposomes exposed to TX 100 (A) and naked dex-HEMA nanogels (B,C,D) stored in PB at 37°C. To prepare the naked dex-HEMA nanogels the SOPC:DOTAP (9:1) lipid film was hydrated with a 20% (w/w) dex-HEMA solution. The DS of the dex-HEMA was respectively 2.5 (B), 5.4 (C) and 8.9 (D). Subsequently the lipid coating was removed by adding TX 100. Repeated experiments revealed the same results.

Uptake of dex-HEMA nanogels by VERO cells

We studied the cellular uptake of the lipid-coated dex-HEMA nanogels. As explained in the Experimental section, for monitoring the cellular uptake the nanogels were fluorescently labeled with Texas Red dextran. To remove free Texas Red dextran (i.e. Texas Red dextran not encapsulated in the nanogels), the nanogel dispersions were filtrated by centrifugational filtration. Separate fluorescence measurements showed that the 70 kDa Texas Red dextran chains could freely pass through the used membrane (molecular weight cut off 100 kDa) while the nanogels (a few hundred nanometer in size) were prevented from passing the membrane, as expected.

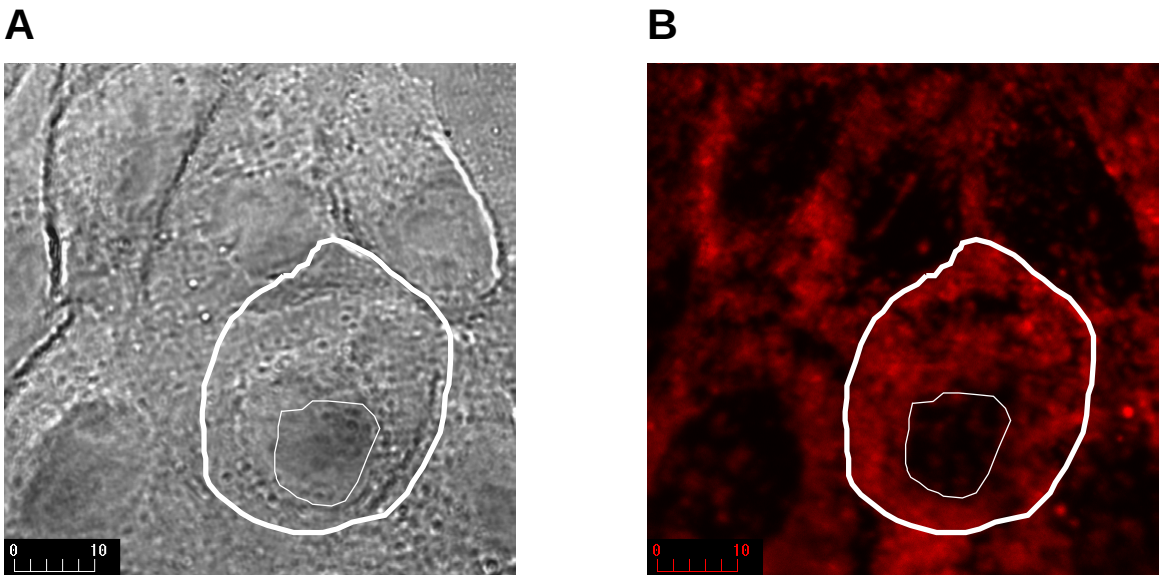

Figure 10. CLSM images of VERO cells incubated with lipid-coated dex-HEMA nanogels (containing Texas Red labelled dextran). To prepare the dex-HEMA nanogels the SOPC:DOTAP (9:1) lipid film was hydrated with a 20% (w/w) dex-HEMA solution (DS 5.4).

Figure 10 shows confocal microscopy images of VERO cells one hour after incubating them with lipid-coated dex-HEMA nanogels: internalization (most likely by endocytosis) of the nanogels clearly occurred as highly fluorescent punctuations were observed in the cytoplasm. Also, the dex-HEMA nanogels seemed to be excluded from the nucleus. This can be expected considering that the pores in the nuclear membrane are by far too small to allow the passage of the (intact/degrading) nanogels[28].

CONCLUSIONS

In this second chapter it is shown that lipid-coated dex-HEMA nanogels can be prepared by UV polymerization of dex-HEMA containing liposomes which were obtained by hydrating a SOPC:DOTAP lipid film with a dex-HEMA solution. DLS measurements indicated that the lipid-coated dex-HEMA nanogels thus obtained were around 350 nm in size, as could be expected considering the 450 nm pores of the membranes used to extrude the liposome dispersions. DLS measurements also clearly showed that TX 100 efficiently removed the lipid coating as the intensity of the light scattered by the dispersions significantly dropped upon adding TX 100. On its turn this was attributed to a lowering of the difference in refractive index between the nanogels and the solvent upon removing the lipid coating. AFM en EM imaging confirmed the existence of the particles and showed that both lipid-coated and naked dex-HEMA nanogels could be obtained.

Compared to e.g. the emulsion polymerization method the use of liposomes to prepare nanogels comprehends several advantages. The size of the nanogels

can be controlled by using membranes with the appropriate pore size in the preparation of the liposomes. Also, organic solvents are not used which is a clear benefit when the nanogels are considered for pharmaceutical purposes (e.g. drug inactivation may occur upon contact with organic solvents). Moreover, and especially, the liposome based method allows the design of both naked nanogels as well as lipid-coated nanogels. The lipid coating may offer additional interesting features to the nanogels. E.g. in the next chapter, we will verify whether the presence of a lipid coating may prevent the 'burst release' of some model drug molecules which will be encapsulated in the hydrogel core. In a following chapter, we will make use of pH sensitive[29-31] and PEGylated lipids to surround the nanogels, as this may improve the escape of the nanogels from endosomes (which is attractive if the nanogels have to be delivered in the cytosol) and improve the particles stability and prolong blood circulation times after parenteral administration. Also, in analogy with immunoliposomes, being liposomes bearing antibodies at their surface, "immunogels" may be easily designed by grafting the antibodies at the lipid surface of the lipid-coated nanogels, making use of well known chemistry.

A major question in this work was whether nanogels could be obtained which degrade over different times. DLS measurements on naked dex-HEMA nanogels stored in PB at 37°C revealed that slowly degrading dex-HEMA nanogels could indeed be realized by increasing the cross-link density of the dex-HEMA nanogels (through the use of dextran densely substituted with HEMA): nanogels prepared with dex-HEMA DS 2.5 were completely degraded in a few days while nanogels synthesized from dex-HEMA DS 8.9 remained to exist, roughly spoken, during two weeks. Our experiments showed that in lipid-coated dex-HEMA nanogels stored over days in buffer at 37°C the dex-HEMA gel also degraded.

As this work showed that the dex-HEMA nanogels could be taken up by endocytosis in VERO cells further research will explore the potentials of the dex-HEMA nanogels for intracellular delivery of antisense therapeutics, plasmid DNA and proteins. In the next chapter, drug loading and release will be in focus as well as the behavior of the nanogels in biological media like serum which may induce aggregation of the nanogels through e.g. the adsorption of proteins at their surface.

ACKNOWLEDGEMENTS

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3

PROTEIN RELEASE FROM BIODEGRADABLE DEXTRAN NANOGELS

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Abstract

The use of drugs with intracellular targets will strongly depend on the availability of delivery systems that are able to deliver them to specific intracellular sites at an optimal rate. In the previous chapter biodegradable dextran nanogels were prepared using liposomes as a nanoscaled reactor. In this chapter we will encapsulate some model proteins (bovine serum albumin (BSA) and lysozyme) in these nanogels. We found that the encapsulation efficiency of BSA and lysozyme in the dextran nanogels was about 50%. Specifically, the release of BSA and lysozyme from the dextran nanogels was clearly governed by the cross-link density of the tiny gels. Depending on the size of the encapsulated protein, the cross-link density of the dextran network and the presence or absence of a lipid coating, proteins were released from the nanogels over days to weeks. Interestingly, when sufficiently diluted, dextran nanogels did not aggregate in human serum, being of major importance when one considers intravenous administration of such nanogels. Also, reconstitution of lyophilized dextran nanogels seemed perfectly possible, being also an important finding since dextran nanogels will have to be stored in dry form.

INTRODUCTION

The successful use of therapeutic molecules with intracellular targets will certainly depend on the availability of delivery systems that deliver an optimal dose of the pharmacologically active agents to specific *intracellular* sites at an optimal rate. There is an emerging need for intracellular delivery vehicles for nucleic acids to be used in gene therapy. Also systems which deliver therapeutic antibodies, peptides and proteins into cells (like for example β-galactosidase, to treat lysozomal storage disease, or apoptosis inducing proteases like caspase 3, caspase 8 and granzyme B) will become of major importance[1]. Liposomes have attracted much attention during the last decade for intracellular delivery of proteins and nucleic acids since they may allow a better targeting of specific cells and may protect the encapsulated drugs from degradation in the blood and the extracellular matrices[2]. Also, liposomes can be made responsive to different stimuli, for example light and pH[3,4], which may hold promise for triggered release of the encapsulated drugs. Generally speaking, liposomes have a low encapsulation efficiency for nucleic acids and protein drugs and they are easily destroyed after injection into the body. Especially, controlling the rate of protein or nucleic acid release from liposomes is not straightforward. Though, in this context we would like to refer to Dalkara et al.[5] who reported on a lipid formulation consisting of *in situ* dimerized CholSpermine (cholesterol linked to carboxy-spermine by a cysteine) and DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) able to enter adherent cells and to release the protein over a prolonged period of time.

In the last few years there has been a significant interest in (biodegradable) polymeric nanoparticles as, compared to liposomes, they are more robust and thus more stable in the body. Some of them show higher encapsulation efficiencies for certain drugs and they can be tailored for time controlled release of drugs. Different types of polymers have been used to design biodegradable polymeric nanoparticles. Poly(lactic-co-glycolic acid) (PLGA) nanoparticles have been extensively investigated[6,7] due to their biocompatibility and biodegradability. GMP grade PLGA is commercially available and it has a long history of safe use in both medical applications (like implants, internal sutures) and drug delivery (like peptide and protein delivery), all features that make PLGA attractive to so many researchers. It is well known, however, that the hydrolysis of PLGA may substantially decrease the pH in PLGA micro- and nanoparticles, which may destroy encapsulated proteins and peptides. But, basic additives like $Mg(OH)_2$ and $Ca(OH)_2$ encapsulated in the PLGA matrix may overcome this[8]. Another disadvantage of PLGA nanoparticles is that the rate of degradation in the body, and thus drug release, is too slow in many cases. Additionally, there is only a limited possibility of modifying the release from PLGA matrices. Besides PLGA, many other types of biodegradable polymers are being investigated to design drug containing nanoparticles, like e.g. poly(cyanoacrylates) (PCA)[9,] chitosan[10], gelatin[11], sodium alginate[12,13].

In chapter 2 we reported on the synthesis of biodegradable dextran nanogels of about 400 nm in size[14]. We showed that lipid-coated dextran nanogels - sometimes referred to as "lipobeads"[15]- can be prepared by UV polymerization of hydroxyethyl methacrylated dextran (dex-HEMA; Figure 1A) containing liposomes which were obtained by hydrating a lipid film with a dex-HEMA solution. "Naked" dextran nanogels (i.e. without lipid coating) were prepared by removing the lipid coating with Triton X 100. Dex-HEMA nanogels spontaneously hydrolyze under physiological conditions into free dextran chains and some poly(HEMA) fragments[16]. DLS measurements on dex-HEMA nanogels stored in buffer at 37°C revealed that the degradation time depended on the cross-link density of the nanogels; dex-HEMA nanogels prepared from dextran sparsely substituted with HEMA degraded quickly while it took days to weeks for nanogels prepared from densely substituted dextran. Furthermore, confocal laser scanning microscopy showed that dex-HEMA nanogels could be taken up by cells.

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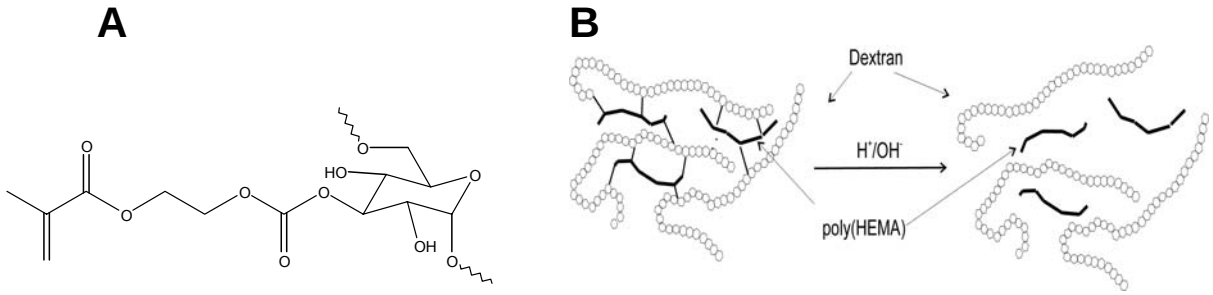

Figure 1. (A) Chemical structure of the monomer in dex-HEMA, i.e. glucopyranose substituted with HEMA and (B) schematic representation of a dex-HEMA network before and after degradation.

In this chapter we investigated whether dextran nanogels can be efficiently uploaded with proteins. Since we know from chapter 2 that the degradation time of the dextran nanogels depends on the cross-link density[14], we evaluated whether we can govern the protein release from such tiny gels by varying their cross-link density. Initially we investigated whether the lipid coating surrounding the dextran nanogels influences the protein release. Finally we studied whether or not dextran nanogels (a) aggregate in serum and (b) keep their physicochemical properties after lyophilization, because dextran nanogels will have to be stored in dry form as moisture degrades them.

EXPERIMENTAL SECTION

Synthesis of dex-HEMA

Dex-HEMA was prepared and characterized as described elsewhere[17]. Dextran with a molecular weight of 19000 g/mol was used in the synthesis of dex-

HEMA. The degree of substitution (DS, i.e. the number of HEMA groups per 100 glucopyranose residues of dextran) was determined by proton nuclear magnetic resonance spectroscopy (^{1}H NMR) in D_2O with a Gemini 300 spectrometer (Varian). The different DS of the dex-HEMA used in this study were respectively 2.5, 5.4 and 8.9.

Preparation of (protein loaded) dextran nanogels

Dextran nanogels were prepared using liposomes as a nanoscaled reactor, as described in the previous chapter[14]. A conventional procedure to prepare the liposomes was used. A 5 mg lipid film of SOPC (1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, Avanti Polar Lipids) was made by dissolving the lipid in chloroform in a test-tube. This solution was dried under a nitrogen flow while gently spinning the vial. This resulted in a thin lipid film on the bottom of the vial. The vial was placed under vacuum for at least 4 hours to remove all remaining chloroform. Subsequently, this dry lipid film was hydrated with 1 mL of a dex-HEMA solution (i.e. 20% (w/w) dex-HEMA in 50 mM phosphate buffer (PB) at pH 7.0) which contained 0.05% (w/w) Irgacure 2959 (Ciba Specialty Chemicals) as a photo initiator. The resulting dispersion was placed at 25°C for 30 minutes while vortexing every 5 minutes. This dispersion of large vesicles was aged overnight. Next, the dispersion was extruded with a LiposoFast Pneumatic-Actuator (Avestin) provided with a 400 nm polycarbonate membrane (Whatman International). After 11 back-and-forth passages of the dispersion through the extrusion membrane, the liposome dispersion was diluted 10 times with PB. This dilution was necessary as not all the dex-HEMA was entrapped in the liposomes. If not diluted, this “free” dex-HEMA would form a gel in the polymerization step, thereby enclosing the liposomes in a polymer matrix. The dispersion was subsequently exposed to UV light (365 nm from a Bluepoint 2.1 UV source, Honle UV Technology) at 25°C for 450 s, which cross-linked the dex-HEMA solution in the liposomes with the formation of “lipid-coated dex-HEMA nanogels”.

To obtain “naked dex-HEMA nanogels” the lipid layer was removed by adding 20.0 µL of a 100 mM solution of the detergent Triton X 100 (TX 100, Merck) to 1 mL of the lipid-coated nanogel dispersion.

To prepare protein loaded dextran nanogels, the protein (bovine serum albumine; BSA; Sigma – lysozyme; Fédération International Pharmaceutique) was added to the dex-HEMA solution used to hydrate the dry lipid film. The protein concentration in the dex-HEMA solution was 50 mg/mL.

Protein encapsulation efficiency in dextran nanogels

To determine the protein encapsulation efficiency the lipid coated dextran nanogels were intensively washed using a stirring cell (Amicon; provided with a 100 nm membrane from Whatman) to remove the non-encapsulated proteins. The

protein content of the waste water after each washing step was measured by a micro-assay procedure[18]. Washing of the nanogels was stopped once the protein content of the waste water came to a minimum.

To determine the protein encapsulation efficiency all the waste fractions were collected and the protein content was measured by HPLC (see details below). Subsequently the lipid coating of the nanogels was removed by adding TX 100 (20 µL of a 100 mM TX 100 solution to 1 mL of nanogels dispersion) and the nanogels were degraded by adding 20.0 µL dextranase (10U/mL) to 1 mL nanogel dispersion. The dextranase was from Sigma (D-1508; diluted to 10 U/ml in 10 mM PB at pH 7.0; one unit delivers 1 µmol of isomaltose per min at pH of 6.0 at 37°C). The protein content of the thus obtained degraded nanogel dispersion was determined by HPLC (see details below). We made sure that dextranase and the degradation products of the dex-HEMA nanogels (being dextran and poly(HEMA) fragments[19] (see Figure 1B)) did not interfere with the detection of BSA and lysozyme by HPLC.

Dynamic light scattering (DLS) analysis on dextran nanogels

Dynamic light scattering measurements were done with a Malvern Autosizer 4700 at a fixed angle of 90°. A He-Ne laser (633 nm, 25 mW) was used as a light source. The phosphate buffer used to dilute the dispersions of the nanogels for DLS measurements was filtered through a 0.1 µm Millipore Durapore® filter. Polystyrene nanospheres (220 ± 6 nm; Duke Scientific Corporation) were used to check the performance of the DLS instrument. The mean hydrodynamic diameter of the particles (d_h) was computed from the intensity-intensity correlation function using the Malvern software package based on the theory of Brownian motion and the Stokes-Einstein equation

$$d_h = \frac{kT}{3\pi\eta D} \qquad \text{eq. 1}$$

where D is the diffusion coefficient (the primary parameter obtained from DLS measurements), k the Boltzmann constant, T the temperature (298 K) and η the viscosity of the solvent (0.96 mPa.s).

To study the degradation of the nanogels in function of (degradation) time, a cuvette was filled with 1.2 mL of the nanogel dispersions and sealed with Parafilm™ to avoid contamination by dust particles. The cuvette was placed at 37°C and DLS measurements on the degrading nanogels were performed at regular intervals.

Protein release from degrading dextran nanogels

The amount of protein released from the degrading dextran nanogels was measured as schematically presented in Figure 2. A Vivaspin centrifugation filtration device (having a membrane with a 300 kDa MWCO, Vivascience) was filled with a known weight (at least 2,5 g) of a dispersion of protein loaded dextran nanogels in

PB (Figure 2A). Before the nanogels began to degrade and at different times during their degradation (which occurred at 37°C), the amount of released protein was determined by centrifugation of the dispersion for 3 minutes (at 100×g, Figure 2B). The filtrate was stored for protein analysis till the end of the experiment (at 4°C, it was then verified that no degradation of the protein had occurred). PB was added to the nanogel dispersion (remaining in the Vivaspin device) until reaching the original weight of the dispersion (Figure 2C and D). The protein release was measured until the nanogels were completely degraded, as could be detected by DLS measurements.

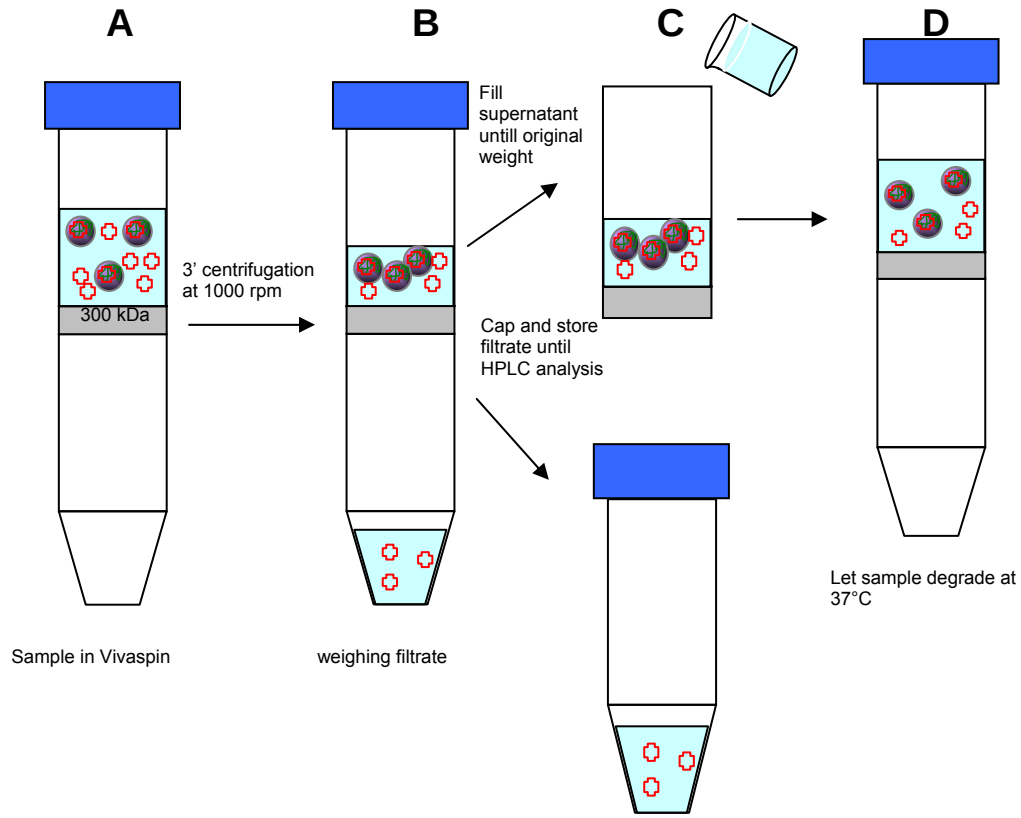

Figure 2. Schematic representation of protein separation process from degrading nanogels.

Determination of the protein concentration

The protein concentration was measured by Reversed Phase HPLC (RP-HPLC, LaChrom Elite). An aliquot (2 µl) of the samples was automatically injected into an RP-HPLC system (Hitachi LaChrom Elite, L-2100 SMASH pump, L-2200 autosampler, L-2300 column oven and L-2450 Diode Array Detector). An RP C4 column (Alltech, 300Å, 5 µm, 25 cm) was used. A water/acetonitrile mixture (the solvent's composition and the concentration gradient in function of time are given in Table 1A and B), adjusted to pH 2.0 with trifluoroacetic acid (TFA, Sigma) was used as the mobile phase at a constant flow rate of 0.75 mL/min. A calibration curve was obtained by injecting various volumes of a 5 mg/mL protein solution in PB. The

protein concentration in the samples was calculated from the area under the protein peak, using the calibration curve.

Table 1. Eluents' (A) composition and (B) concentration gradient used in the mobile phase measuring the protein concentration with HPLC.

A

component	solvent A	solvent B
Water	95 % (v/v)	5% (v/v)
Acetonitrile	5% (v/v)	95% (v/v)
TFA	1 mL/L	1 mL/L

B

time (min)	% solvent A	% solvent B
0	80	20
15	50	50
20	0	100
21	80	20
40	80	20

Enzymatic activity of released lysozyme

The enzymatic activity of lysozyme released from the nanogels was measured by monitoring the clearance of a *Micrococcus luteus* suspension (lysozyme degrades the outer cell wall of these bacteria). For comparison, the same *M. luteus* suspension was treated with a lysozyme standard with well known activity[20]. To 3.00 mL of a buffered *M. luteus* suspension, 10.0 µL of sample was added and mixed at 25°C. The absorbance at 450 nm was read with a spectrophotometer (Pharmacia Biochrom 4060 UV-Visible spectrophotometer) every 15 s for 3 min. The activity of the sample was calculated as follows:

$$a = \frac{S \times c_{st} \times a_{st}}{S_{st} \times c} \qquad \text{eq. 2}$$

Where a is the activity of the sample, S is the absorbance decrease per minute (as calculated from the initial linear part of the absorbance ~ time curve) and c is the concentration of the sample. S_{st}, c_{st} and a_{st} refer to the lysozyme standard, S,

c and a refer to the unknown sample which contains lysozyme released from the nanogels. For kinetic reasons the unknown lysozyme sample was diluted to give an absorbance decrease per minute of the same order of magnitude as the lysozyme standard.

Behavior of dextran nanogels in serum

A Venosafe Serum-Gel tube with cloth activator (6 mL) was filled with human blood. Serum was obtained after blood clotting for 30 min at room temperature, followed by centrifugation at 3000 rpm for 8 min. Subsequently the serum was filtered through a 2 µm filter (Whatman International).

A lipid-coated dextran nanogel dispersion (prepared with dex-HEMA DS 5.4) was added to prewarmed serum (37°C) and further incubated at 37°C. At regular time intervals DLS measurements were performed.

Lyophilization of dextran nanogels

Dextran nanogels with and without SOPC coating were prepared as described above, however, instead of PB, the same amount of distilled water was used. The dextran dispersions were dialyzed at 4°C (to retard/avoid degradation of the nanogels) for one week in a 100 fold volume of water. Subsequently the dispersions were weighed and sucrose was added (final sucrose concentration was 10% (w/w)). Then the dispersions were freeze-dried (Amsco-Finn Aqua GT4 freeze –dryer) as follows: The dispersions were frozen to 228 K within 175 min at 1000 mbar. Primary drying was performed at 258 K and at a pressure varying between 0.8 and 1 mbar for 13 h, followed by secondary drying at an elevated temperature (283 K) and reduced pressure (0.1-0.2 mbar) for 7 h.

RESULTS AND DISCUSSION

Synthesis of protein loaded dextran nanogels using liposomes as reactor

In chapter 2 we reported that both lipid-coated and ‘naked’ dex-HEMA nanogels can be prepared by UV polymerization of dex-HEMA containing liposomes which were obtained by hydrating a lipid film with a dex-HEMA solution[14]. We wondered whether protein filled dextran nanogels could also be obtained in this manner. Figure 3A shows the typical outcome of DLS measurements on a liposome dispersion obtained by hydrating a SOPC lipid film with a dex-HEMA/lysozyme solution. The size of the liposomes is around 400 nm, well in agreement with the pore size of the extrusion membrane used. Figure 3B shows DLS measurements on the same liposomes but after UV treatment that cross-links the dex-HEMA in the interior of the liposomes. One can see no aggregates are formed. It therefore

seems possible to prepare (rather sparsely dispersed) dextran nanogels using liposomal vesicles as reactor when proteins are present in the dex-HEMA solution which hydrates the lipid film. In the process of their formation liposomes passively entrap water and these dissolved molecules being dex-HEMA and proteins in this study.

As described in the Experimental section, the non-encapsulated proteins and non-encapsulated dex-HEMA were washed away from the nanogels by the use of a stirring cell. Figure 3C shows DLS measurements on "washed" dextran nanogels; clearly, after washing, intact dextran nanogels, approximately 400 nm in size, remain present. In the filtrate we could not detect any nanogels by DLS, as expected, proving that the nanogels could not pass the filter of the stirring cell.

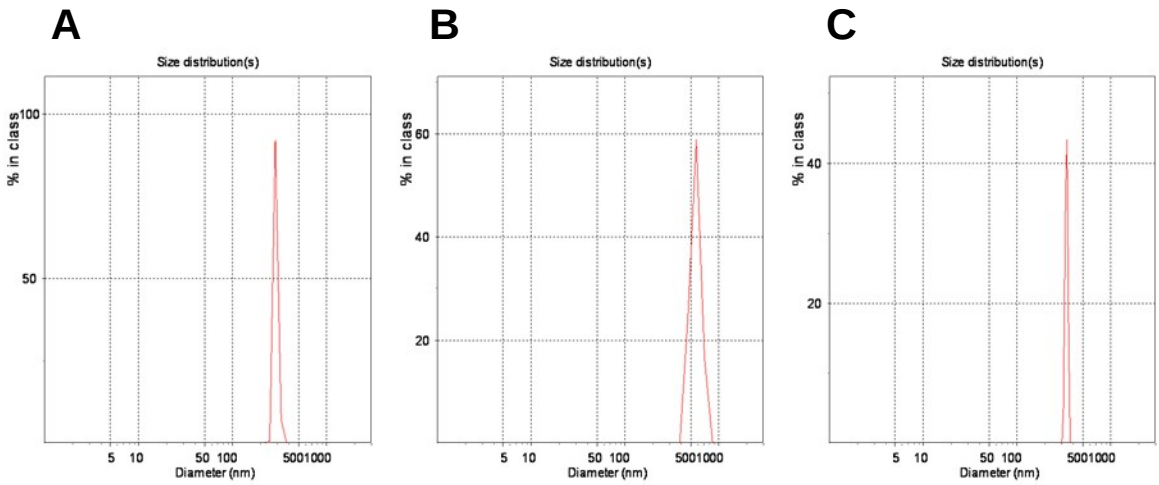

Figure 3. Size distribution, as measured by DLS, of SOPC coated dex-HEMA nanogels loaded with lysozyme. A 20% (w/w) dex-HEMA (DS 5.4) solution was used to hydrate the SOPC lipid film. (A) shows the SOPC liposomes before polymerization of the dex-HEMA, (B) and (C) show the SOPC coated dex-HEMA nanogels respectively before and after 3 washing steps.

Figure 4 shows the outcome of protein measurements on the filtrate. As one can see, most of the non-encapsulated proteins were removed from the nanogels after two washing steps, since the absorbance by the filtrate after two washing steps is not higher than the absorbance by the blank (PB 50 mM pH 7.0). In all further experiments, the dextran nanogel dispersions were washed three times.

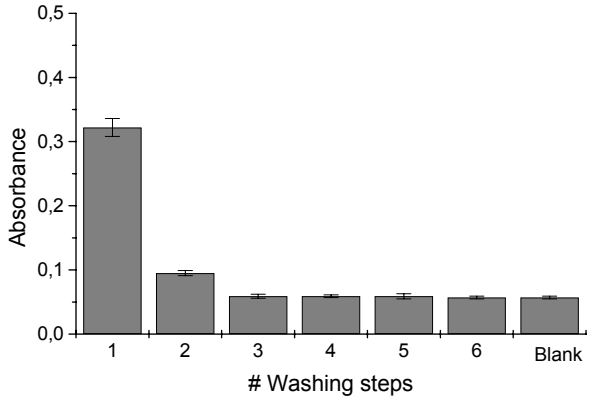

Figure 4. Protein absorbance of the filtrate after washing a dex-HEMA nanogel dispersion several times (DS 5.4; SOPC coated, BSA loaded nanogels). As blank PB (50 mM, pH 7.0) was used.

Encapsulation of proteins in dextran nanogels

To determine the loading efficiency of dextran nanogels with proteins we determined both the amount of protein encapsulated in the nanogels as well as the amount of non-encapsulated protein (i.e. the amount of protein in the washing water). The former was determined after removal of the lipid coating of the dextran nanogels and digestion of the nanogels with dextranase. The removal of the lipid is necessary since we observed in the previous chapter that dextranase could not penetrate through the lipid layer[14]. Table 2 shows that about half of the proteins are entrapped in the nanogels. As expected, the protein loading was not dependent on the cross-link density of the nanogels since the loading occurs before the cross-linking of the dex-HEMA solution in the liposomes. Note that the "total amount of protein", being the sum of the encapsulated and non-encapsulated amount, is somewhat lower than expected (i.e. 50 mg per sample) which is, highly likely, due to some protein loss during the experiment. Because the non-coated nanogels were made from the lipid-coated ones, we assume that the protein loading of the naked dextran nanogels equals the loading of the corresponding lipid-coated nanogels.

Table 2. Protein encapsulation in SOPC coated nanogels.

Protein	DS dex-HEMA	Removed with washing water (mg)	Encapsulated in the nanogels (mg)	**Encapsulated in the nanogels (%)**
BSA	2.5	24	22	**48**
	5.4	21	23	**52**
	8.9	20	22	**51**
lyso	2.5	26	22	**45**
	5.4	25	22	**46**
	8.9	24	21	**46**

Protein release from (hydrogel free) liposomes

A major objective of this study was to evaluate whether the dextran network in the nanogels allows controlling the release of encapsulated proteins. For this purpose we first studied what the release of proteins from (hydrogel free, i.e. without dex-HEMA) liposomes looks like. Figure 5 clearly shows that about 20% of the encapsulated BSA was initially released from SOPC liposomes. No further release occurred during the next 18 days; showing that BSA remains encapsulated in the SOPC liposomes. Note that a 100% release in Figure 5 corresponds to the amount of BSA measured after treatment of the liposomes with TX 100. In contrast to BSA, lysozyme is released almost immediately (Figure 5). While the reason is unclear, the fact that lysozyme is much smaller than BSA, may play a role. The molecular weight and hydrodynamic diameter of BSA are 67000 g/mol and 7.2 nm while they are 12000 g/mol and 4.1 nm for lysozyme[21].

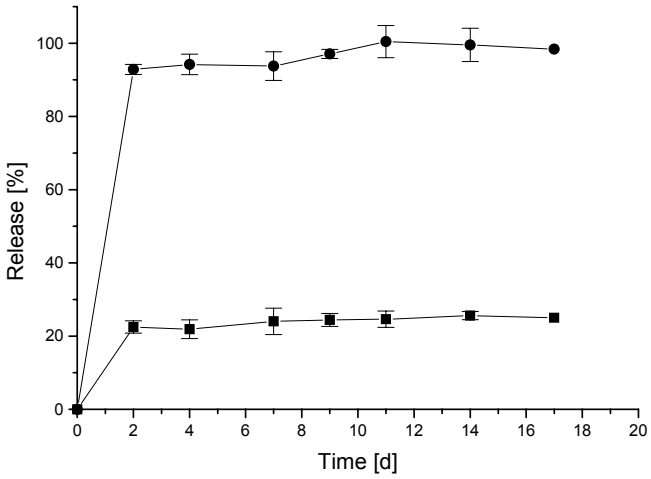

Figure 5. Relative cumulative release of BSA (■) and lysozyme (●) from (hydrogel free) SOPC liposomes in function of time. 100% release corresponds to the amount of protein measured after adding TX 100 to the liposomes.

Protein release from dextran nanogels

DLS measurements on dex-HEMA nanogels stored in buffer at 37°C described in chapter 2 revealed that the degradation time depends on the cross-link density of the nanogels: dex-HEMA nanogels prepared from dextran sparsely substituted with HEMA degrade quickly while it takes days to weeks for nanogels prepared from densely substituted dextran[14]. We now wondered whether varying the cross-link density of the dextran nanogels influences the release of the encapsulated proteins.

Measuring drug release *in vitro* from nanoparticles is always a challenge because the nanoparticles must be separated from the release medium[22]. For that purpose ultra-centrifugation is often used[23-25]. This is a rather time consuming

process which occurs at high centrifugal forces, which may destroy the nanoparticles, especially since nanogels become softer and softer as degradation proceeds. We separated the dextran nanogels from the released proteins by centrifugational filtration as described in the Methods and Materials section. The separation process was optimized to make sure the nanogels remained quantitatively on the filter and that the protein concentration on top of the filter equaled the concentration in the filtrate.

Figure 6 represents the release of BSA from sparsely (DS 2.5; A), moderately (DS 5.4; B) and densely (DS 8.9; C) cross-linked dextran nanogels, respectively with and without SOPC lipid coating. As we know from chapter 2, the "life-time" of the naked dextran nanogels (i.e. the time to become completely degraded under the conditions of the release experiment and as measured by DLS) is around respectively 5 days for DS 2.5, 12 days for DS 5.4 and 18 days for DS 8.9[14].

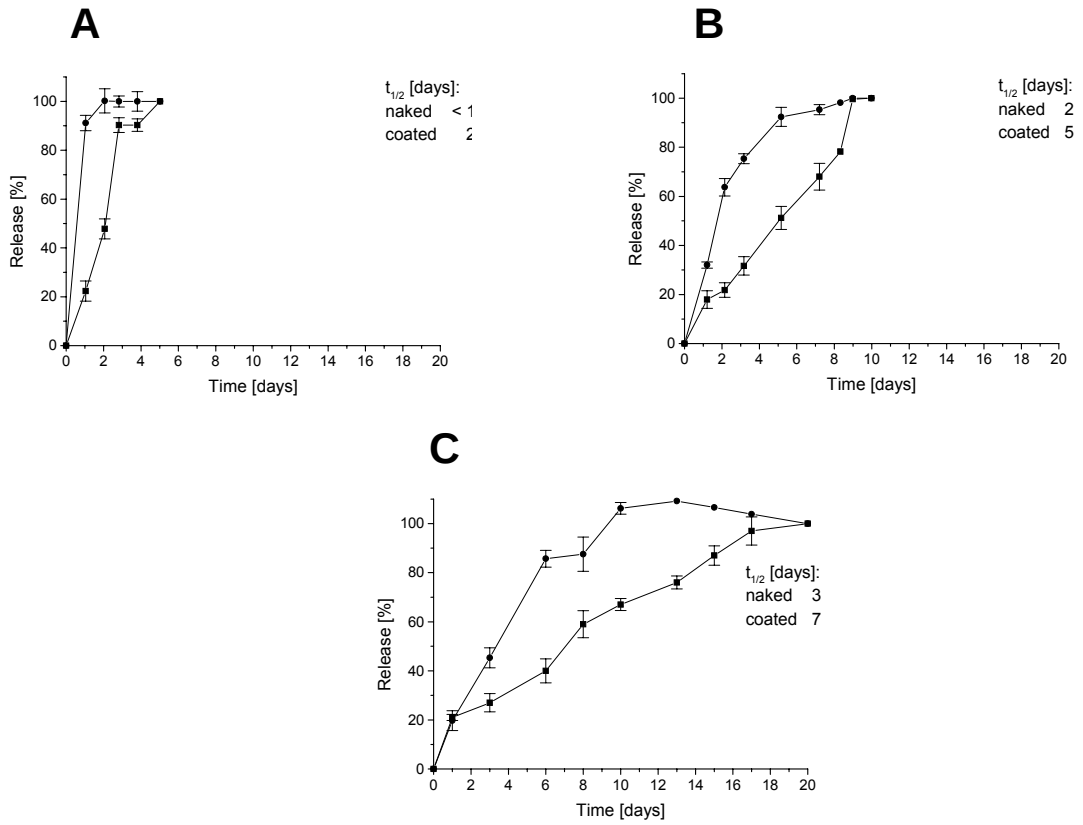

Figure 6. Cumulative release of BSA from degrading SOPC coated (■) and naked (●) dex-HEMA nanogels. In all experiments a 20% (w/w) dex-HEMA solution was used to hydrate the lipid film. The DS of the dex-HEMA was 2.5 (A), 5.4 (B) and 8.9 (C). 100% release corresponds to the amount of BSA released when the nanogels were completely degraded (i.e. when nanogels could no longer be detected by DLS).

Let us first consider the BSA release from the *naked* nanogels. Clearly, the cross-link density of the dextran nanogels has an important influence on the release of the proteins. The insets in Figure 6 tabulate the time it takes to release approximately 50% of the proteins ($t_{1/2}$). For the sparsely cross-linked nanogels

(Figure 6A) $t_{1/2}$ is shorter than 1 day while $t_{1/2}$ is 2 to 3 days for the DS 5.4 and 8.9 dextran nanogels (Figure 6B and C, respectively). This suggests that BSA molecules are initially physically entrapped in the dex-HEMA network of the nanogels. The higher the cross-link density, the more cross-links have to be hydrolyzed to sufficiently enlarge the pores of the network thereafter allowing the proteins to be released.

Compared to the naked nanogels, the lipid-coated dextran nanogels release BSA more slowly (Figure 6). An effect of the lipid coating on the BSA release could be somehow expected, as BSA remains encapsulated in SOPC liposomes for longer times (see Figure 5). The question arises as to why BSA is rather continuously released from the lipid-coated dextran nanogels. To explain this, the following considerations may be important. First, we showed in chapter 2 that dex-HEMA nanogels in the liposomes degrade, just like the naked dex-HEMA nanogels do[14]. Indeed, after keeping lipid-coated dextran nanogels for a sufficiently long time in PB (to allow degradation) and after removing the remaining lipid coating (by TX 100), nanogels were no longer detected. Second, as was also observed for dex-HEMA gel slabs and dex-HEMA microgels[26,27], dex-HEMA nanogels very likely swell during degradation. Third, our research group observed previously that the turnover from dextran gels into a solution of the degradation products increases their osmotic pressure; provided the gels are surrounded by a semi-permeable membrane, which allows water transport, but keeps the free dextran chains in the degrading matrix[26,28,29]. Considering these three observations we hypothesize that the degrading (swelling) dextran nanogel cores deform their surrounding lipid coatings thereby making them leaky to proteins. As a result, BSA may be continuously delivered from the lipid-coated nanogels, rather than remaining in the vesicles. On the other hand, another possibility could be that the particles completely loose their lipid coating before they can release the included proteins. However, this is highly unlikely because it is shown in chapter 2 that the lipid coat stays around the particles during degradation.[14]

Figure 7 shows the release of lysozyme from degrading dextran nanogels. The following observations can be made. First, in contrast to the release of BSA, the release of lysozyme from lipid-coated and naked dextran nanogels occurs similarly. In other words, the lipid coating does not retard the release of lysozyme. This could be expected as Figure 5 showed that lysozyme does not stay within SOPC liposomes suggesting that the SOPC lipid membrane is fully permeable to lysozyme. Second, lysozyme is released faster from dextran nanogels than BSA, and this occurs in the sparsely, moderately as well as the densely cross-linked nanogels. This could be explained by the fact that lysozyme is a smaller protein than BSA (the calculated hydrodynamic diameters are respectively 4.1 nm and 7.2 nm[30]). Third, as was also seen for the release of BSA, there is an influence of the network structure.

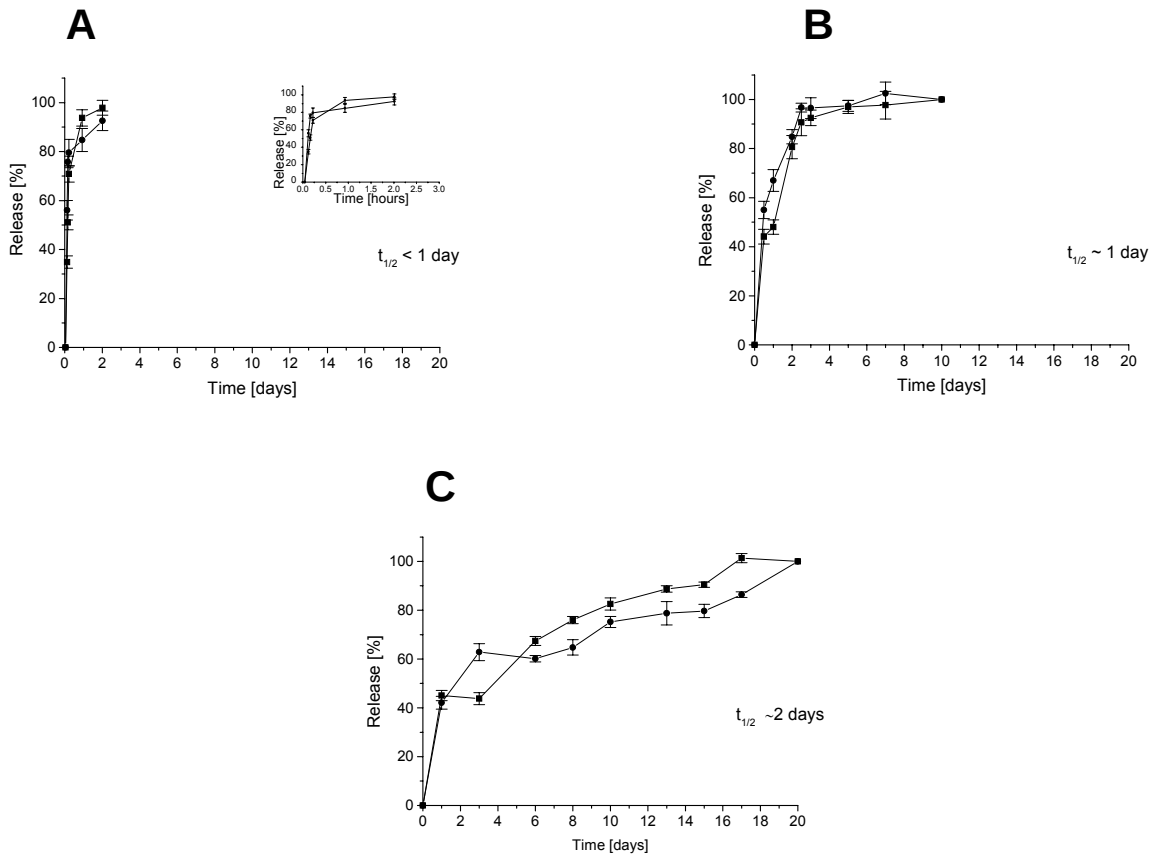

Figure 7. Cumulative release of lysozyme from degrading SOPC coated (■) and naked (●) dex-HEMA nanogels. In all experiments a 20% (w/w) dex-HEMA solution was used to hydrate the lipid film. The DS of the dex-HEMA is 2.5 (A), 5.4 (B) and 8.9 (C). 100% release corresponds to the amount of lysozyme released when the nanogels were completely degraded (i.e. when nanogels could no longer be detected by DLS).

Comparing Figure 7A to C, one can see that the lysozyme release from a more densely cross-linked dextran network is slower than from a sparsely cross-linked dextran network. This shows that lysozyme is physically entrapped in the polymer network of the nanogels; cross-links have to be degraded before the entrapped lysozyme can escape.

Enzymatic activity of released lysozyme

It is of great importance that proteins do not loose their biological activity due to the encapsulation process, thus keeping their tertiary and quaternary structure. Indeed, the main disadvantage of photopolymerization comes from the necessary production of highly reactive free radicals that initiate the cross-linking. These free radicals may induce side reactions between the encapsulated drug molecules and polymer chains. As an example, Quick et al.[31-33] used photopolymerized hydrogels for DNA encapsulation and delivery. They showed that without the presence of monomer, free radicals attack and damage the DNA molecules. Chun et al.[34] came

to similar conclusions; DNA entrapped in Pluronic™ hydrogels made by UV polymerization was gradually damaged with increasing UV exposure time. Leach et al.[35] included BSA into glycidyl methacrylate-hyaluronic acid hydrogels by photopolymerization and showed that protein aggregates were formed.

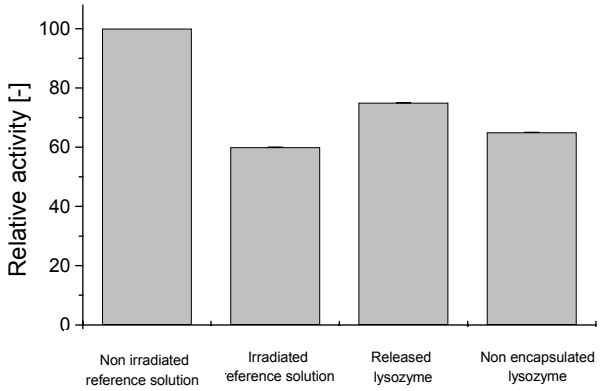

Figure 8. Enzymatic activity of lysozyme after different treatments. The activity of a non-irradiated lysozyme solution was set to 100. The enzymatic activity of each sample was normalized against the lysozyme concentration of the sample.

Enzymes are ideal model proteins to test whether encapsulated proteins do not become destroyed during the photopolymerization step as even minimal structural changes may lower their catalytic activity. For this purpose we tested whether lysozyme encapsulated in dextran nanogels keeps its enzymatic activity. The activity of lysozyme was monitored by absorbance measurements on a *M. luteus* suspension, as described in the Experimental Section. The results are shown in Figure 8. The activity of a non-UV irradiated lysozyme solution was set as 100%. The activity of non-encapsulated lysozyme, as separated from the dextran nanogels by the use of the stirring cell, was measured as well. The activity of the lysozyme released from the dextran nanogels (kept at 37°C for several days until they were completely degraded, and checked by DLS) was also measured. The enzymatic activity of each lysozyme sample was normalized to the lysozyme concentration in the sample. As Figure 8 shows, the activity of the released lysozyme was 75% of the activity of the non-UV irradiated reference solution. We also measured the enzymatic activity of the reference lysozyme solution after irradiation in the presence of the photo initiator to determine the effect of UV-induced radicals on the activity of lysozyme. Two things can be seen in Figure 8; the UV irradiation significantly lowered the activity, and that the activity of non-encapsulated lysozyme is somewhat lower than the activity of released lysozyme. This could possibly be explained by the fact that radicals in the liposome container are primarily used for activation of the double bonds on dex-HEMA which would keep the lysozyme molecules more protected. Although the results on lysozyme are promising, one should realize that

other peptides or proteins may undergo structural changes during the photopolymerization step.

Dextran nanogels in human serum

Clearly, after intravenous administration dextran nanogels should never be allowed to aggregate since micron sized particles cannot pass through narrow (lung) capillaries. We evaluated *in vitro* how lipid-coated dextran nanogels behave in human serum. DLS measurements on nanoparticles in human serum are not evident, because serum components (like proteins) strongly scatter the light. Aggregated structures were indeed detected by DLS in serum (i.e. before the dextran nanogels were added; data not shown) and therefore the serum was filtered through a 2.0 µm filter before adding the nanogels. The outcome of DLS measurements on the filtered serum is represented in Figure 9A while Figure 9B shows the outcome of DLS measurements on nanogels in PB. Subsequently we mixed human serum and a nanogel dispersion in respectively 5:5 and 9:1 volume ratios. In case of the 5:5 ratio (part) of the nanogels aggregated immediately as micron sized particles were detected (Figure 9C). After 5h the 400 nm-sized particles could even no longer be detected, only particles in the µm range were seen (Figure 9E). When much more serum was used in the dilution, (volume ratio 9:1) the nanogels did not seem to aggregate (Figure 9D); not even after being in serum for 5h (Figure 9F). The results in Figure 9 suggest that, very likely, dextran nanogels will not aggregate after intravenous injection since the injected nanogel dispersion will become very diluted in the bloodstream.

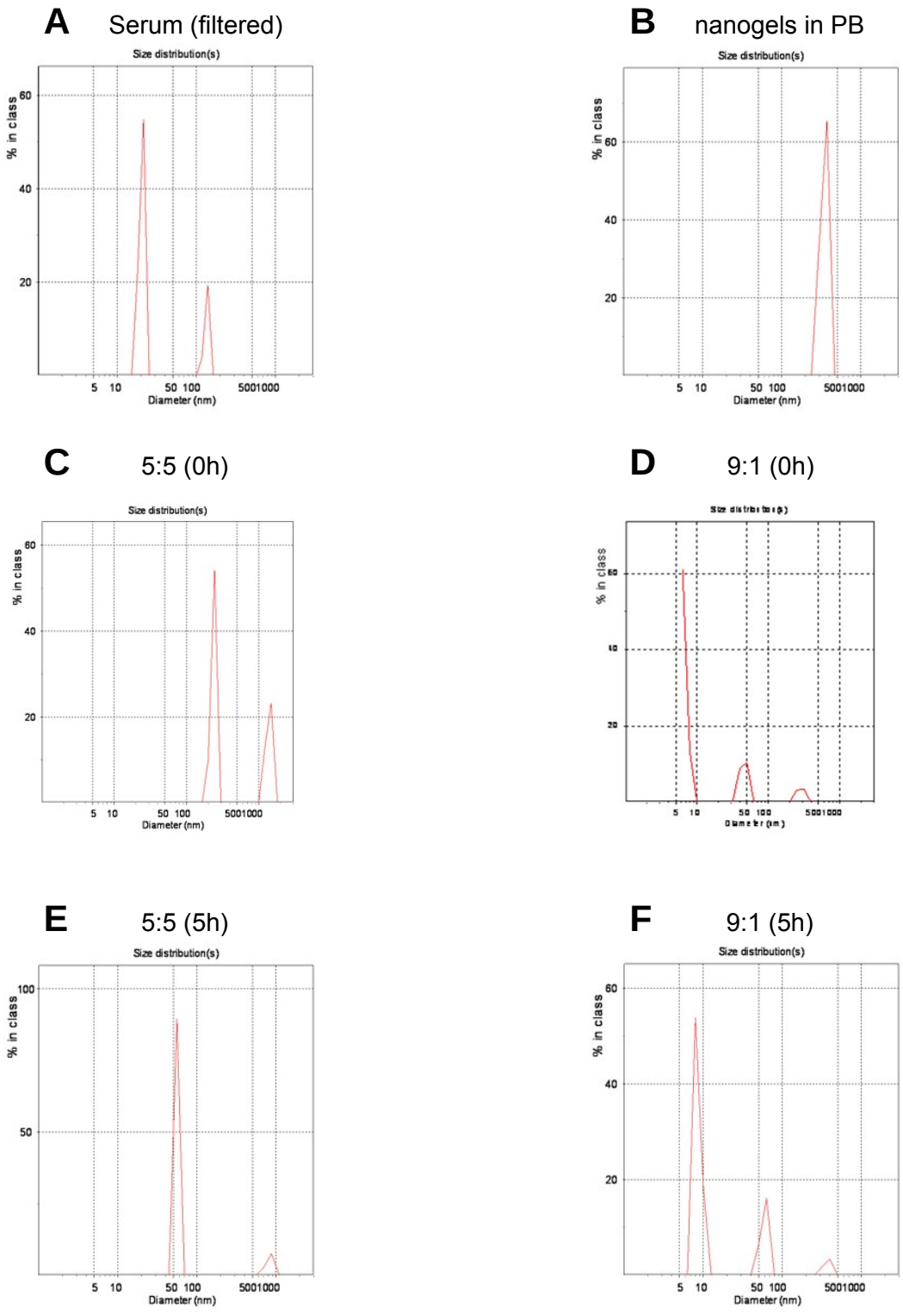

Figure 9. Size distribution, as measured by DLS, of (A) human serum after filtration through a 2.0 µm filter; (B) SOPC coated dex-HEMA nanogels (DS 5.4) in PB pH 7.0. SOPC coated dex-HEMA nanogels in (filtered) human serum (in a 5:5 volume ratio) immediately (C) and 5 h (E) after mixing. SOPC coated dex-HEMA nanogels in (filtered) human serum (in a 9:1 volume ratio) immediately (D) and 5 h (F) after mixing.

Lyophilization of dextran nanogels

We next evaluated (a) whether it is possible to freeze dry lipid-coated and naked dextran nanogels and (b) whether reconstituted lyophilized nanogels still show the same degradation behavior as the original, non-lyophilized samples. Particles were lyophilized in the presence of 10% (w/w) sucrose as a cryoprotectant. After lyophilization, the dry dextran nanogels were reconstituted in PB reaching the original weight (i.e. the weight of the sample before lyophilization). The reconstituted nanogels were incubated at 37°C and measured by DLS at regular time intervals

Figure **10**A and B show the results for respectively the naked and lipid-coated nanogels. The following observations were made. First, after reconstitution dextran nanogels were still detected. Second, the lyophilized naked dextran nanogels degraded similarly to the non-lyophilized ones, which is an important finding. It suggests that the lyophilization process does not change the physicochemical properties of the naked dextran nanogels. Third, Figure 10B indicates that both the lyophilized and the non lyophilized lipid-coated nanogels show the same behavior: it seems that the lipid-coated nanogels do not disappear as a function of time.

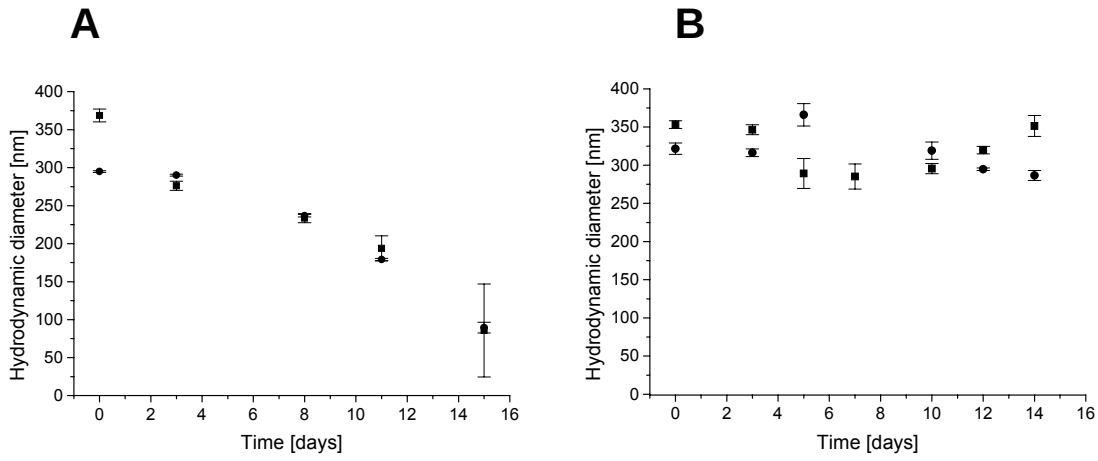

Figure 10. DLS measurements on freeze-dried (■) and non freeze-dried (●) nanogels without (A) or with (B) SOPC coating. In between measurements, the nanogels were stored in PB at 37°C. To prepare the dex-HEMA nanogels, the SOPC lipid film was hydrated with a 20% (w/w) dex-HEMA solution. The DS of the dex-HEMA was 5.4. Data shown are the mean of five size measurements on one same sample.

At first sight one could conclude that the dex-HEMA nanogels in the liposomes do not degrade. However, we showed in the previous chapter that the hydrolysis of the dex-HEMA nanogels in the liposomes does occur. When TX 100 was added to a dispersion of lipid-coated dextran nanogels that were allowed to degrade for 12 days, nanogels were no longer detected[14]. This proved that the nanogels in the liposomes, the non-lyophilized as well as the lyophilized ones were indeed completely degraded into a dextran and poly(HEMA) solution.

CONCLUSIONS

In this third chapter it is shown that dextran nanogels, which are prepared by UV polymerization of dex-HEMA containing liposomes, are promising carriers for the encapsulation and controlled delivery of proteins. We found the encapsulation efficiency of BSA and lysozyme in the dextran nanogels to be about 50%. Especially, the release of BSA and lysozyme from the dextran nanogels was clearly governed by the cross-link density of the tiny gels. Depending on the size of the encapsulated protein, the cross-link density of the dextran network and the presence or absence of a lipid coating, proteins were released from the nanogels over days to weeks. Interestingly, when sufficiently diluted, dextran nanogels did not aggregate in human serum, being of major importance considering the intravenous administration of such nanogels. Also, reconstitution of lyophilized dextran nanogels seemed perfectly possible, being also an important finding since dextran nanogels will have to be stored in dry form.

We would like to stress that compared to the emulsion polymerization method, the use of liposomes to prepare nanogels has several advantages. The size of the nanogels can be controlled by using membranes with the appropriate pore size in the preparation of the liposomes. Also, organic solvents are not used which is a clear benefit when the nanogels are considered for the encapsulation of proteins that may inactivate upon contact with organic solvents. Moreover, and especially, the liposome-based method allows the design of both naked nanogels as well as lipid-coated nanogels. The lipid coating may offer at least three additional interesting features to the nanogels. One, the use of pH sensitive lipids[36-38] may improve the escape of the nanogels from endosomes which is attractive if the nanogels have to be delivered in the cytosol. Two, in analogy with immunoliposomes, being liposomes bearing antibodies at their surface, “immunogels” may be easily designed by grafting the antibodies at the lipid surface of the lipid-coated nanogels, making use of well known chemistry. And three, by using PEGylated lipid coatings, the circulation time of the nanogels may be prolonged[39-41].

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4

DEXTRAN NANOGELS WITH FUNCTIONAL COATINGS: PEG-LIPID-COATED AND PH SENSITIVE LIPID-COATED NANOGELS

Abstract

The aim of this chapter was to design PEGylated lipid coated dextran nanogels as an intracellular drug delivery system. Additionally, when the particles are in an acidic environment, for example in the endosome, the particles must loose their coating

DOTAP:DOPE liposomes containing DSPE-PEG_{2000} (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000) were used as a nanoscopic mould to cross-link dex-HEMA. To obtain particles with a pH sensitive lipid layer, an acid sensitive lipid, diplasmenylcholine, was formulated into the liposome. The size and charge of the particles and the interaction with serum was evaluated by DLS and zèta potential measurements. The interactions with cells were visualized by confocal microscopy.

It was shown that lipid coated dextran nanogels with a PEG modified surface are about 450 nm in size and bear almost no charge. Although the cellular binding is lower than non-PEGylated particles, cellular uptake both by VERO and retinal pigment epithelial cells was equally efficient. When the particles were mixed with human serum in equal amounts, aggregation is observed, however at higher dilutions, the particle suspension was stable for at least 7 hours.

As it is well known that PEGylated particles often do not escape from the endosomal compartment and end up in the lysosomes were they are degraded. We wanted to make nanogels that lose their PEGylated coating in the endosome, after which they can escape to the cytosol. It was shown that the incorporation of an acid sensitive lipid into the lipid coating, resulted in the removal of the PEGylated coating at lower pH.

INTRODUCTION

Appropriately designed materials should improve the intracellular delivery of large molecules such as peptides, oligonucleotides and plasmids. PEGylation of nanoscopic drug carriers is a widely used technique since it improves their stability and prolongs the blood circulation time[1-3]. Biological entities such as serum consist of numerous components including lipids and proteins, causing non-specific adsorption on the nanosphere surface[4]. To improve the non-fouling characteristics of the particles, PEGylation is often used as PEG creates a steric barrier against interactions with molecular and cellular components in the biological environment[5,6]. Besides stability, PEGylation also prolongs the blood circulation time of drug carriers by inhibition of opsonin proteins binding to the particles' surface. These proteins allow macrophages of the mononuclear phagocytic system (MPS) to easily recognize and remove drug delivery devices before they can perform their desired therapeutic function[7]. A sufficient blood circulation time is of great importance for drug delivery because the required therapeutic level of pharmaceuticals can be achieved only when the carriers stay in the blood stream for an appropriate time. If this is achieved, the nanoparticles will accumulate in pathological sites with affected and leaky vasculature (such as tumours, inflammations and infarcted areas) due to the enhanced permeability and retention (EPR) effect[8]. PEGylated liposomes have been extensively investigated for delivery of many therapeutic molecules, for example doxorubicin, docetaxel, paclitaxel, vasoactive intestinal peptide and different cytokines[9-13]. Although promising, liposomes may not be the best candidates for sustained drug delivery because of their relatively limited drug loading capacity and short release duration of entrapped drugs[14].

Hydrogels are more stable and higher loading efficiencies can eventually be achieved. For that reason, in this chapter we wanted to develop nanoscopic dextran hydrogel particles surrounded by a PEG-modified lipid surface as a long-circulating intracellular delivery system. In chapter 2 we showed that it is possible to produce nanogels using liposomes as a nanoscopic mould, following a procedure described by Kazakov et al.[15] A solution of a polymerizable dextran -i.e. dextran-hydroxyethylmethacrylate (dex-HEMA, Figure 1A)- was captured inside the cavity of a liposome and polymerized by UV irradiation, resulting in a dextran nanogel surrounded by a lipid layer[16]. Dex-HEMA is composed of a dextran backbone substituted with polymerizable HEMA groups and the corresponding hydrogel degrades spontaneously under physiological conditions into dextran and some poly(HEMA) fragments (Figure 1B). The degradation time depends on the polymer concentration and the degree of substitution (DS, i.e. the number of HEMA groups per 100 glycopyranose residues)[17,18].

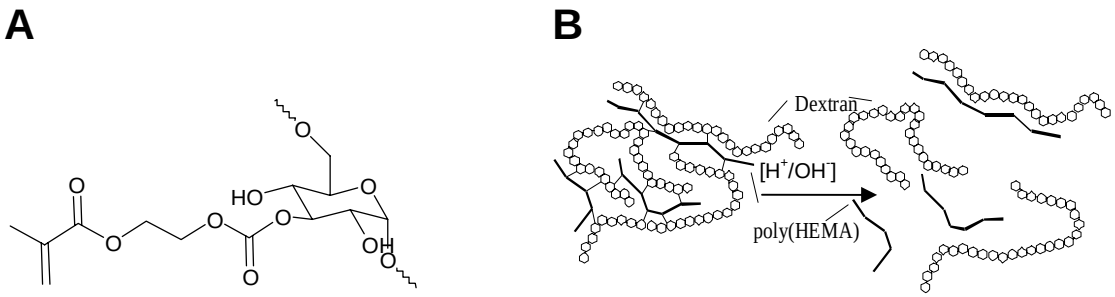

Figure 1. (A) Chemical structure of the monomer in dex-HEMA, i.e. glucopyranose substituted with HEMA. (B) Schematic representation of the dex-HEMA polymer network before (left) and after (right) degradation. The HEMA cross links degrade through hydrolysis of the carbonate esters, resulting in dextran chains and poly(HEMA).

It was shown that the degradation time of these nanogels could be altered from a few days to some weeks and depends on the DS. In chapter 3[19], we showed that nanogels can be loaded with proteins simply through the addition of the desired protein to the starting polymer solution. Depending on the size of the encapsulated protein, the cross-link density of the dextran network and the presence or absence of a lipid coating, proteins were released from the nanogels over days to weeks. The loading efficiency is about 50% and -in spite of the UV treatment of the nanogels in the presence of proteins- lysozyme kept about 75% of its activity. Moreover, the nanogels can be freeze-dried without change in degradation profile, offering possibilities for long-term storage [19].

As described above, the nanogels are made using liposomes as nanoscopic "reaction containers". To produce PEGylated nanogels, we will use PEGylated liposomes as a mould for polymerization and fill them with a dex-HEMA solution. After polymerization, lipid covered nanogels are produced, surrounded by a PEG "halo" and filled with a dex-HEMA hydrogel holding drug molecules, as schematically represented in Figure 2.

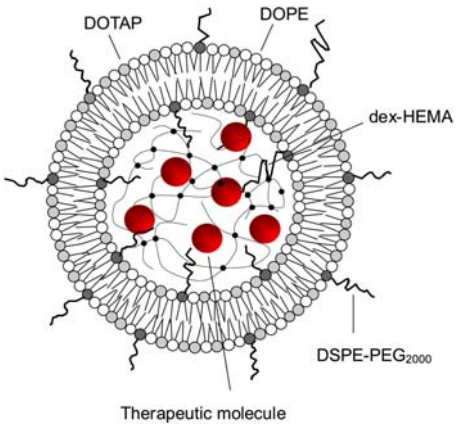

Figure 2. Schematic representation of nanogels filled with a therapeutic molecule and surrounded by a PEGylated lipid layer.

A major problem with PEGylated particles is their retention in the endosome followed by lysosomal degradation. As a result, the bioavailability and efficacy of included drugs is often minimal[20]. Particles that lose their PEGylated lipid coating upon arrival in the endosome would be a solution to this issue. The intrinsic low pH within endosomal environments offers an appropriate trigger to detach the lipid layer from the nanogel. The acid labile lipid coating falls apart at the acidic environment of the endosome, leaving naked (non-PEGylated) nanogels that can escape easily into the cytoplasm. One of the first studies concerning the inclusion of PEG lipids in pH sensitive *liposomes* was performed by Slepushkin et al.[21] and contained DOPE:CHEMS:PE-PEG (DOPE being 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine, CHEMS is cholesteryl hemisuccinate and PE-PEG is a PEG coupled lipid). These polymer modified liposomes show fusion properties at acidic conditions and transfer their content into the cytoplasm by fusing with the endosomal membrane. Although the development of sterically stabilized pH-sensitive liposomes has been associated frequently with the incorporation of DOPE in the liposome formulation[22-28], other strategies have been examined[29-32].

Nanoscopic particles surrounded by a lipid coating -also referred to as lipobeads[15]- are already produced for diverging purposes such as drug delivery[33-37], biosensors[38-41] or artificial blood substitutes[42,43]. Lipid coated nanogels with a pH sensitive *core* were developed by Kazakov et al.[15]; the poly(N-isopropylacrylate-vinyl imidazole) core was surrounded by an egg phosphatidylcholine (EPC) coating, but the pH sensitivity was demonstrated only on naked nanogels -i.e. without the surrounding lipid layer. To the best of our knowledge, no lipid coated hydrogel particles have been produced with both a PEGylated and pH sensitive *coat*.

Rui et al.[44] reported on the acid-catalyzed hydrolysis of synthetic diplasmenylcholine: 1,2-di-O-(Z-1'-hexadecenyl)-*sn*-glycero-3-phosphocholine (DPPlsC, Figure 3). The electron-rich vinyl ether linkage in this lipid is susceptible to acid catalyzed hydrolysis, resulting in glycerophosphocholine and single-chain surfactant products.

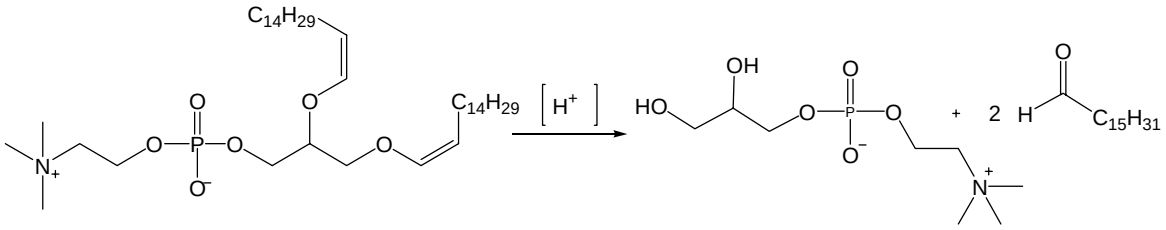

Figure 3. 1,2-di-O-(Z-1'-hexadecenyl)-sn-glycero-3-phosphocholine (DPPlsC), a pH sensitive lipid for the synthesis of a pH sensitive lipid membrane. At low pH degradation occurs into glycerophosphocholine and hexadecanoal.

As a result, liposomes containing DPPlsC are stable at physiological pH (pH 7.4) but undergo degradation at endosomal pHs[45]. We wanted to investigate whether it is possible to create lipid coated dextran nanogels using both pH sensitive and PEGylated liposomes as a mould for the polymerization reaction. This would result in PEGylated lipid coated nanogel that loses its surrounding lipid layer at low pH values (Figure 4).

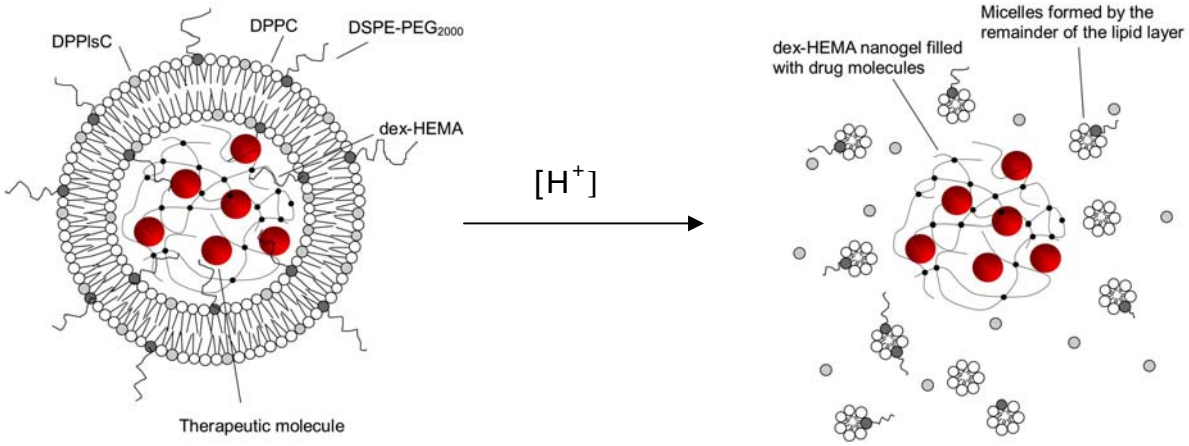

Figure 4. Schematic representation of a dex-HEMA nanogel, containing therapeutic molecules and surrounded by a pH sensitive (PEGylated) lipid-coating. At pH 4.0, the lipid-coating comes of, resulting in a naked nanogel and micelles.

So briefly, in this chapter we will examine whether it is possible to prepare dextran nanogels with a PEGylated lipid coating, we will examine their serum stability and their cellular binding and uptake properties. We will also try to produce dextran nanogels with a pH sensitive and PEGylated lipid coating, being detached from the nanogels at endosomal pH.

EXPERIMENTAL SECTION

Dex-HEMA Preparation and Characterization.

Dex-HEMA batches were prepared and characterized according to a method described elsewhere[46]. Dextran (Fluka, from *Leuconostoc ssp*.) with a number average molecular mass of 19000 g/mol was used. The degree of substitution was determined by proton nuclear magnetic resonance spectroscopy (^{1}H NMR) in D_2O with a Gemini 300 spectrometer (Varian). The DS of the dex-HEMA used in this study was 5.4.

Preparation of dextran nanogels

Dextran nanogels were prepared using liposomes as a nanoscaled reactor, as described in the second chapter[16]. We prepared the liposomes using a standard

procedure. A 5 mg lipid film of DOTAP:DOPE (1:1 molecular ratio, resp 1,2-Dioleoyl-3-Trimethylammonium-Propane and 1,2-dioleoyl-sn-Glycero-3-Phosphoethanolamine, Avanti Polar Lipids) was made by dissolving the lipid in chloroform in a test-tube. This solution was dried under a nitrogen flow while gently spinning the vial. This resulted in a thin lipid film which was placed under vacuum for at least 4 hours to remove all remaining chloroform. Subsequently, this dry lipid film was rehydrated with 1 mL of a dex-HEMA solution (i.e. 20% (w/w) dex-HEMA in 50 mM phosphate buffer (PB) at pH 7.0) containing 0.05% (w/w) Irgacure 2959 (Ciba Specialty Chemicals) as a photo initiator. The resulting dispersion was placed at 25°C for 30 minutes while vortexing every 5 minutes. This dispersion of large vesicles was aged overnight and was extruded with a LiposoFast Pneumatic-Actuator (Avestin) provided with a 400 nm polycarbonate membrane (Whatman International). After 11 back-and-forth passages of the dispersion through the extrusion membrane, the liposome dispersion was diluted 10 times with PB. This dilution was necessary because not all the dex-HEMA was entrapped in the liposomes. If not diluted, this "free" dex-HEMA would form a gel in the polymerization step, thereby enclosing the liposomes in a polymer matrix. The dispersion was subsequently exposed to UV light (365 nm from a Bluepoint 2.1 UV source, Honle UV Technology) at 25°C for 450 s which cross-linked the dex-HEMA solution in the liposomes with the formation of "lipid coated dex-HEMA nanogels". To obtain "naked dex-HEMA nanogels" the lipid layer was removed by adding 20.0 µL of a 100 mM solution of the detergent Triton X 100 (TX 100, Merck) to 1 mL of the lipid coated nanogel dispersion.

To obtain PEGylated nanogels, 5 mol% of DSPE-PEG_{2000} (1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-2000] (Ammonium Salt), Avanti Polar Lipids) was added to the starting lipid/chloroform solution.

For the preparation of nanogels with a PEGylated pH sensitive lipid coat, the starting lipid film was composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, Avanti Polar Lipids) and 1,2-di-O-(Z-1'-hexadcenyl)-sn-glycero-3-phosphocholine (DPPlsC) in a 8:2 molar ratio with 5 mol% DPSE-PEG_{2000}. This pH sensitive lipid is presented in Figure 3 and was synthesized as described by Van den Bossche et al.[47] The liposome dispersion was obtained by hydrating the lipid film with 1 mL PB at a temperature at least 10°C above the phase transition temperature (i.e. at 45°C) during 30 min and vortexing every 5 min. Extrusion was performed at the same temperature.

Dynamic light scattering (DLS) and zèta potential analysis on dextran nanogels

Phosphate buffer, filtered through a 0.1 µm Millipore Durapore® filter, was used to dilute the dispersions of the nanogels to study the hydrodynamic diameter (d_h) and zèta potential (ζ) of the nanogels. A cuvette was filled with 1.2 mL of the

nanogel dispersions and sealed with Parafilm™ to avoid contamination with dust particles. Polystyrene nanospheres (220 ± 6 nm; Duke Scientific Corporation) were used to check the performance of the DLS instrument. The size distribution and the zèta potential of the degrading particles were determined by use of a Zetasizer Nano ZS (Malvern Instruments).

Cell lines and cellular uptake of nanogels

African green monkey kidney (VERO-1) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) at 37°C containing 2 mM glutamine, 10% heat-inactivated fetal bovine serum and 1% penicilline-streptomycine. Cells were prophylactically treated against mycoplasma with Plasmocin (Invivogen). The retinal pigment epithelial cell line (D407) [48] was a kind gift from Dr. Richard Hunt (University of South Carolina, Medical School, Columbia, USA). The cells were cultured in DMEM with phenol red. The cells were seeded onto sterile glass bottomed culture disks (MatTek Corporation), allowed to grow and to adhere for 1 day (at 37°C in a humidified atmosphere containing 5% CO_2) before applying the nanogel dispersions.

Cell binding experiments

For evaluation of the cellular binding and uptake of the dex-HEMA nanogels (DS 5.4), the nanogels were fluorescently labeled with Texas Red labeled dextran (70 kDa, Molecular Probes). 60 µL of a Texas Red dextran solution (25 mg/mL) was added to 1 mL of dex-HEMA/photo initiator solution used to hydrate the lipid film. Removal of free Texas Red dextran (i.e. not entrapped in the liposomes) was achieved with Microcon™ centrifugal filter devices (molecular weight cut off 100 kDa, Millipore Corporation). After centrifugation (at 14000×g for 24 minutes), the fluorescent dex-HEMA filled liposomes were recovered by inverted spin and diluted ten times with DMEM before UV polymerization. DLS measurements showed no remarkable change in size after centrifugational filtration (data not shown).

For cell binding experiments, the cells were incubated for 15 minutes at 4°C with the labelled nanogels. The culture medium was removed and replaced by 200 µL cold (4°C) DMEM, containing lipid coated nanogels labeled with Texas Red Dextran. The cells were incubated for another 15 minutes at 4°C before microscopy experiments were performed.

Cell internalization experiments

For cell internalization experiments, the nanogels were labelled with Texas Red dextran as described above. 200 µL of lipid coated nanogels (containing Texas Red labeled dextran and prewarmed to 37°C) were added to the cells and incubated for 1 h at 37°C. The cells were washed three times with PB before imaging by confocal laser scanning microscopy. Confocal Laser Scanning Microscopy (CLSM,

Nikon C1si) images were taken using a Nikon 60× oil immersion objective and a 561 nm DPSS laser for the excitation of the Texas Red labeled nanogels.

Behavior of dextran nanogels in (human) serum

A Venosafe Serum-Gel tube with cloth activator (6 mL) was filled with human blood. Serum was obtained after blood clotting for 30 min at room temperature, followed by centrifugation at 3000 rpm for 8 min. Subsequently the serum was filtrated trough a 2.0 µm filter (Whatman International). The lipid coated dextran nanogel dispersion (prepared with dex-HEMA DS 5.4) was separated from the surrounding buffer using Microcon™ centrifugal filter devices. Prewarmed serum (37°C) was added and further incubated at 37°C. At regular time intervals DLS and zèta potential measurements were performed.

RESULTS

Synthesis of PEGylated nanogels

To produce PEGylated nanogel using liposomes as a nanoscaled mould, the starting lipid film was composed of DOTAP:DOPE with 5 mol% of a PEGylated lipid, i.e. DSPE-PEG_{2000}. The lipid film was hydrated with a 20% (w/w) dex-HEMA (DS 5.4) solution and the size distribution was measured by DLS. As shown in Figure 5A there is hardly any difference in size between the PEGylated and non-PEGylated liposomes filled with a dex-HEMA solution. Also the cross-linking of this polymer solution inside the liposomes has no effect on the size distribution of both type of particles (Figure 5B). The zèta potential on the other hand differs significantly (Figure 5C): non-PEGylated nanogels have a zèta potential of about 50 mV while the PEGylated nanogels have an almost neutral zèta potential (i.e. 8 mV).

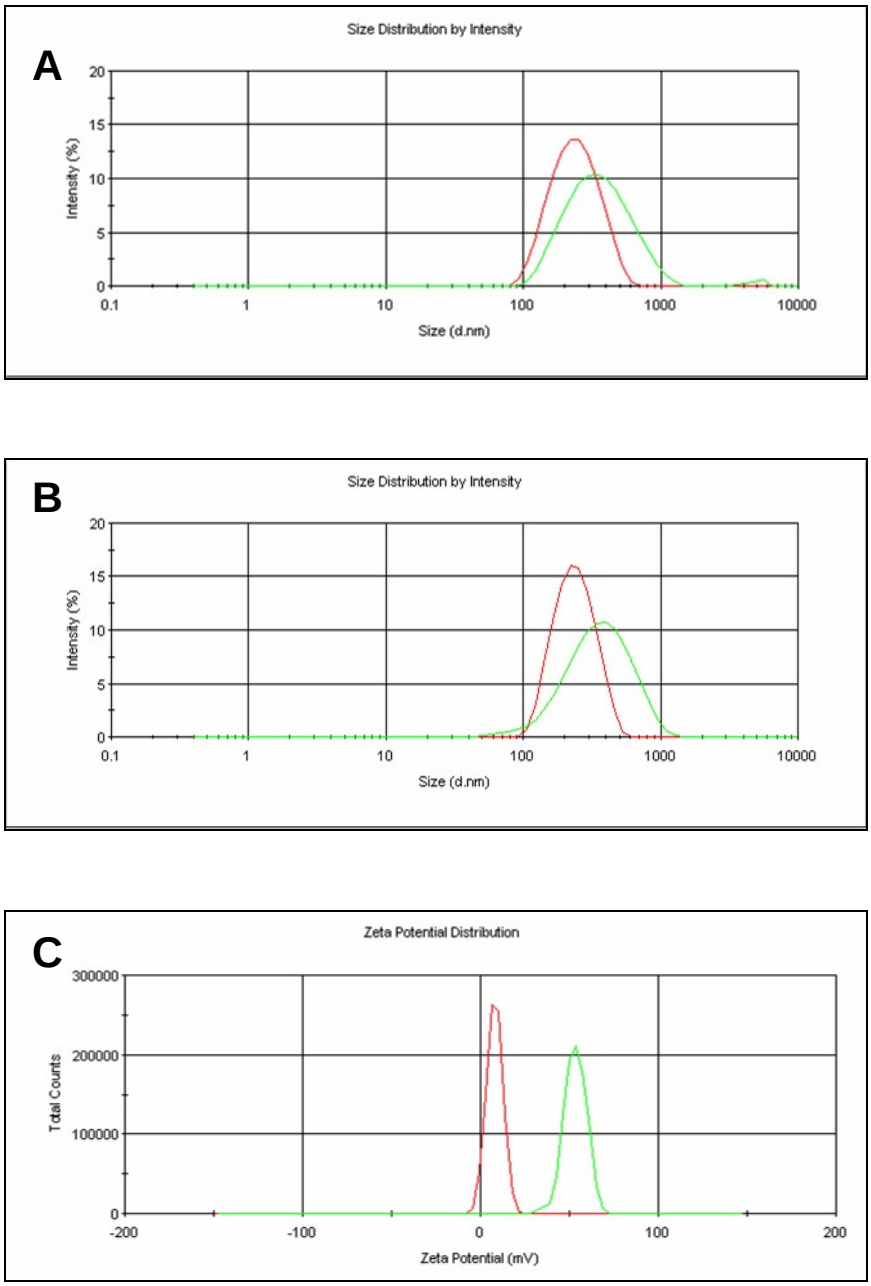

Figure 5. Size distribution, as measured by DLS, of (—) DOTAP:DOPE and DOTAP:DOPE:DSPE-PEG_{2000} (—) coated dex-HEMA nanogels. A 20% (w/w) dex-HEMA (DS 5.4) solution was used to hydrate the lipid film. (A) and (B) show the nanogels before respectively after polymerization of the dex-HEMA; (C) shows the zèta potential of both types of nanogels.

Cellular uptake of PEGylated nanoparticles

Cellular binding and uptake of the particles was monitored using confocal laser scanning microscopy. The nanogels were fluorescently labeled with Texas Red dextran. The free Texas Red dextran (i.e. Texas Red dextran not encapsulated in the nanogels) was removed by centrifugational filtration. Separate fluorescent

measurements showed that the 70 kDa Texas Red dextran chains could freely pass through the membrane (molecular weight cut off 100 kDa) while the nanogels (a few hundred nanometer in size) were prevented from passing through the membrane (data not shown).

Cell binding experiments. The cells were incubated with nanogels at 4°C, which avoids cellular internalization. Figure 6A and C show that PEGylated particles exhibit little membrane association to respectively RPE and VERO cells. Non-PEGylated particles on the other hand bind very well to the cells' surface (Figure 6B for RPE cells and Figure 6D for VERO cells).

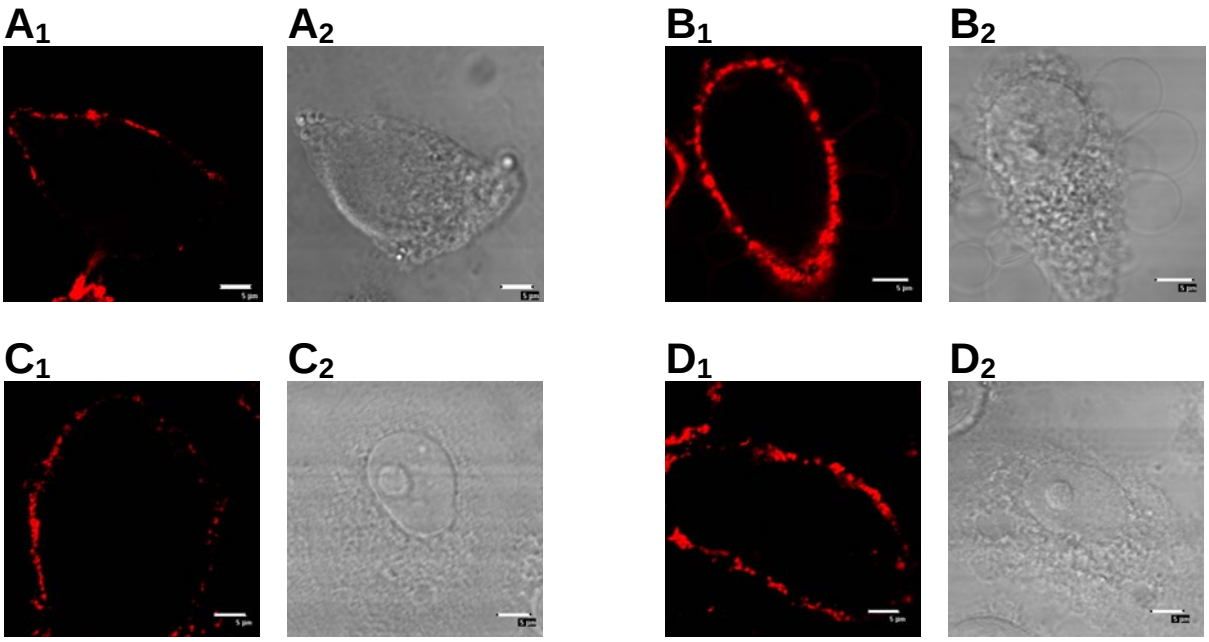

Figure 6. Microscopy images of an RPE cell incubated at 4°C for 15 minutes with (A) PEGylated and (B) non-PEGylated DOTAP:DOPE coated dex-HEMA nanogels. (C) and (D) show a VERO cell incubated at 4°C for 15 minutes with PEGylated and non-PEGylated DOTAP:DOPE coated dex-HEMA nanogels respectively. Subscript 1 and 2 refer respectively to the CLSM image and the transmission image. To prepare the dex-HEMA nanogels the lipid films were hydrated with a 20% (w/w) dex-HEMA solution (DS 5.4) containing Texas Red labelled dextran. Scale bars represent 5µm.

Cell internalization experiments. Figure 7A and C show confocal images of respectively RPE and VERO cells after incubation of dextran nanogels with a PEGylated lipid coating during 1 hour at 37°C. Fluorescent punctuations in the cytoplasm, equally distributed throughout the cytoplasm, in both types of cells are observed. However, they are excluded from the nucleus. As a control experiment, we administered non-PEGylated particles to RPE and VERO cells and as expected from former studies[16] they are taken up as well (Figure 7B and D respectively).

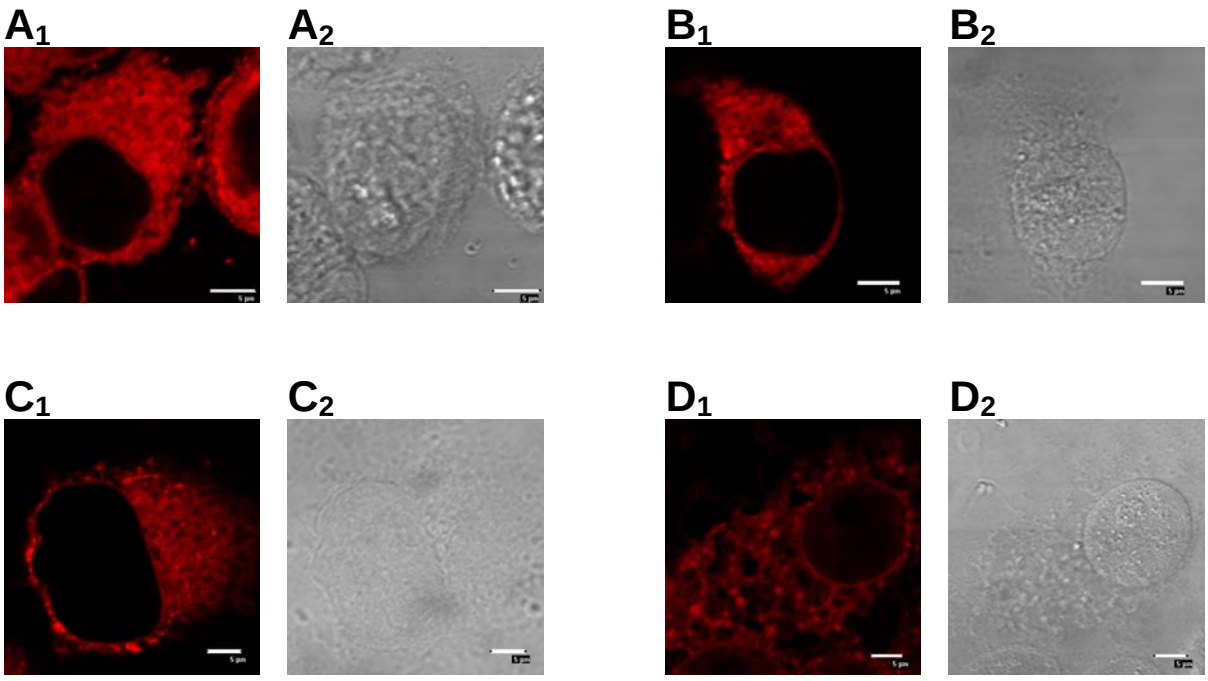

Figure 7. Microscopy images of an RPE cell incubated at 37°C for one hour with (A) PEGylated and (B) non-PEGylated DOTAP:DOPE coated dex-HEMA nanogels. (C) and (D) show a VERO cell incubated at 37°C for one hour with PEGylated and non-PEGylated DOTAP:DOPE coated dex-HEMA nanogels respectively. Subscript 1 and 2 refer respectively to the CLSM image and the transmission image. To prepare the dex-HEMA nanogels the lipid films were hydrated with a 20% (w/w) dex-HEMA solution (DS 5.4) containing Texas Red labelled dextran. Scale bars represent 5µm.

Stability of PEGylated nanoparticles in human serum

The outcome of DLS measurements on the filtered serum is represented in Figure 8A. Figure 8B and C show 100 µL of nanogel suspension mixed with 900 µL of human serum, kept at 37°C and measured 7h after mixing. It is clearly shown that both the PEGylated as the non-PEGylated particles are stable during this period, because 300nm sized particles - coming from the nanogels- can be separately distinguished from the particles present in the serum (particles < 100 nm, see Figure 8A). When the nanogel suspension and serum were mixed in equal amounts, aggregation occurs, as micron sized particles can be detected (Figure 8D and E).

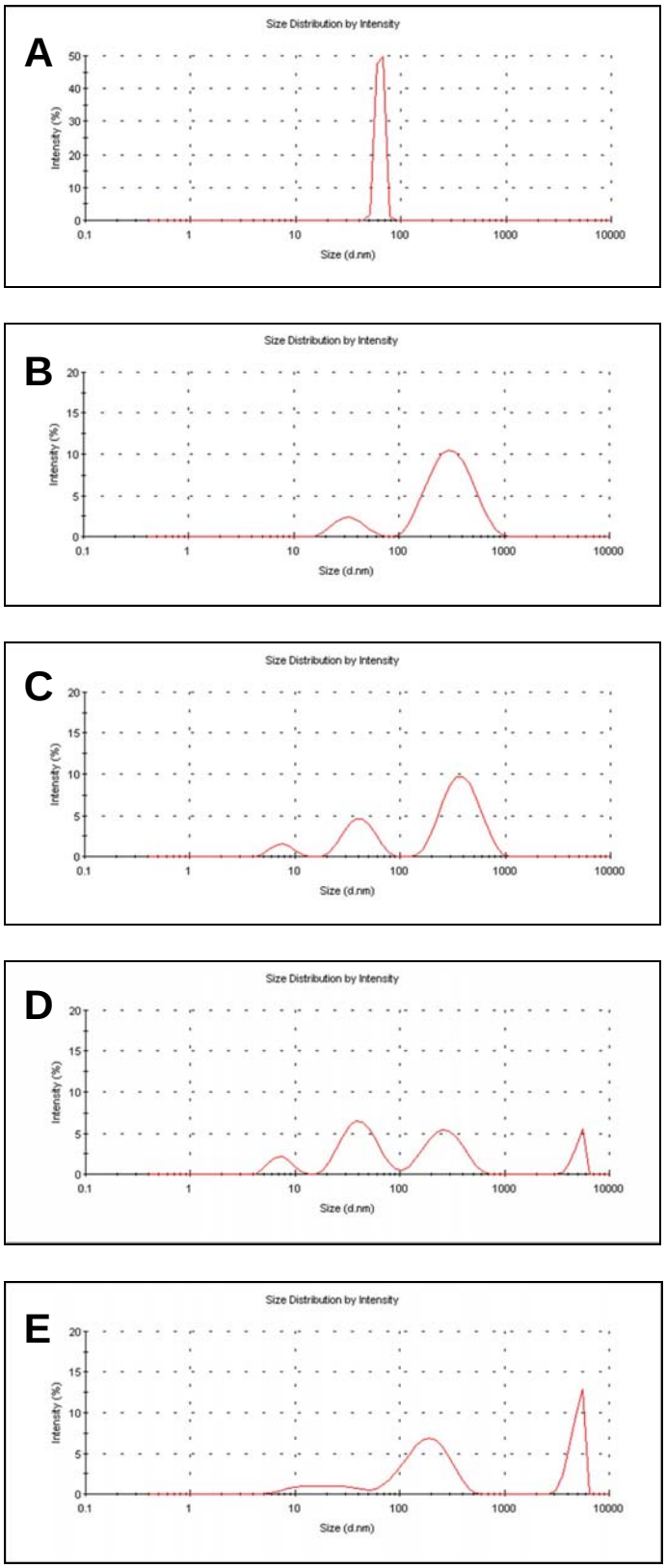

Figure 8. Size distribution, as measured by DLS, of (A) human serum after filtration through a 2.0 µm filter; DOTAP:DOPE:DSPE-PEG_{2000} (B) and DOTAP:DOPE (C) coated dex-HEMA nanogels in (filtrated) human serum (in a 1:9 volume ratio); DOTAP:DOPE:DSPE-PEG_{2000} (D) and DOTAP:DOPE (E) coated dex-HEMA nanogels in (filtrated) human serum (in a 1:1 volume ratio). Measurements were performed 7 h after mixing.

Synthesis of pH sensitive nanogels

Figure 9A and B show that pH sensitive and pH insensitive nanogels do not differ in size at pH 7.4, before or after polymerization: in both cases particles of about 350 nm in size could be detected. At pH 4,0 the pH sensitive nanogels show not only a peak at 350 nm, but also much smaller particles (~12 nm) were detected (Figure 9C). 12 nm is the typical size of micelles, in this case formed by DPPC, DSPE-PEG_{2000} and degradation products of DPPlsC (see 1D). The control nanogels, i.e. nanogels with a DPPC coating containing 5 mol% DSPE-PEG_{2000}, do not show the appearance of such a peak. To make sure that the 12 nm peak is coming from the remainder of the lipid layer, TX 100 was added to both dispersions to solubilize the lipid coating. A peak of 12 nm could be detected for the pH sensitive as well as the pH insensitive nanogels, coming from micelles composed of TX 100 and lipid molecules.

Another indication for the removal of the lipid layer was the significant drop of the intensity of the light scattered by the dispersion which could even be seen visually. Figure 10 shows a picture of nanogel dispersions at different conditions. At pH 7.4 both types of dispersions are opaque because the surrounding lipid layer scatters the incident light (Figure 10 cuvette 1 and 2). At pH 4.0, the lipid coating of the pH sensitive nanogels is removed. As a result, the scattering of the light is lowered due to the refractive index difference between the (aqueous) nanogels and the surrounding aqueous medium is almost zero, resulting in a transparent suspension of nanogels (Figure 10 cuvette 3). The pH insensitive nanogel dispersion is still opaque because the lipid layer stays around the nanogels (Figure 10 cuvette 4). When the lipid layer is removed by use of a detergent, all nanogel dispersions are transparent (Figure 10 cuvette 5 and 6).

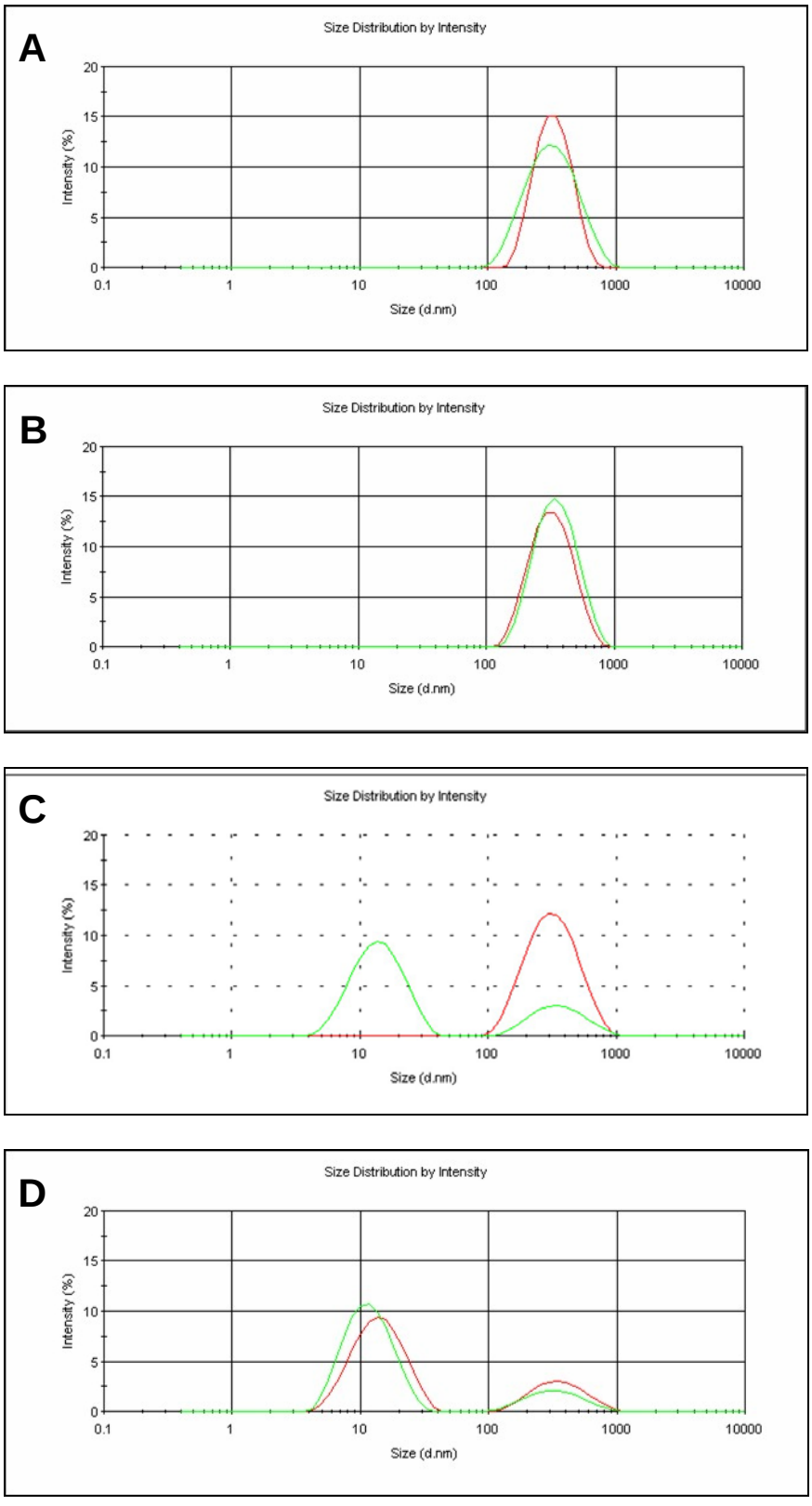

Figure 9. Size distribution, as measured by DLS, of (—) DPPC:DPPlsC (8:2 molar ratio) containing 5 mol% DSPE-PEG_{2000} and DPPC:DSPE-PEG_{2000} (—) coated dex-HEMA nanogels. A 20% (w/w) dex-HEMA (DS 5.4) solution was used to hydrate the lipid film. (A) and (B) show the nanogels before respectively after polymerization of the dex-HEMA, (C) shows the nanogels at pH 4.0 and (D) shows the nanogels after addition of TX 100 to remove the lipid-coating.

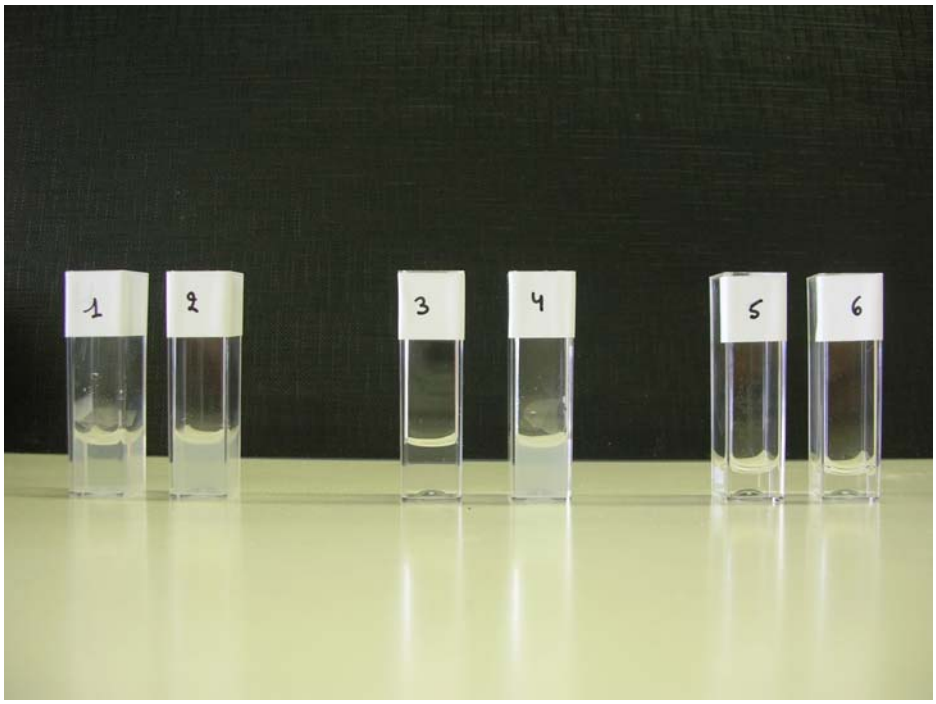

Figure 10. Picture of lipid-coated dex-HEMA (DS 5.4) nanogels. Cuvette 1 and 2 are filled with respectively DPPC:DPPlsC:DSPE-PEG_{2000} and DPPC:DSPE-PEG_{2000} coated dex-HEMA nanogels at pH 7.4. Cuvette 3 and 4 are filled with respectively DPPC:DPPlsC:DSPE-PEG_{2000} and DPPC:DSPE-PEG_{2000} coated dex-HEMA nanogels at pH 4.0. Cuvette 5 and 6 are filled with respectively DPPC:DPPlsC:DSPE-PEG_{2000} and DPPC:DSPE-PEG_{2000}coated dex-HEMA nanogels at pH 7.4 after addition of TX 100. All lipid films were hydrated with a 20% (w/w) dex-HEMA (DS 5.4) solution.

DISCUSSION

The first objective was to determine the possibility to produce PEGylated lipid coated dextran nanogel particles, by use of liposomes as a nanoscopic mould. DOTAP:DOPE liposomes with and without 5 mol% DSPE-PEG were filled with a polymerizable dex-HEMA solution. The nanogels with a PEGylated lipid coating are similar in size when compared to nanogels with a non-PEGylated lipid coating (Figure 5A and B) before and after polymerization. This follows previous data showing that the presence of PEG on the surface of liposomes has no influence on their size[49]. On the other hand, the surface charge of the DOTAP:DOPE:DSPE-PEG_{2000} coated particles is almost neutral, while the DOTAP:DOPE coated particles are positively charged due to the positive charge of DOTAP. The positive charge of DOTAP in the PEGylated nanogels was shielded by the long PEG tales stretching out from the particles' surface.

We know from chapter 2 that it is possible to administer lipid coated nanogels to VERO cells[16], cells with a very good endocytotic capacity. As PEGylation changes the physicochemical properties of the particles' surface, we wanted to investigate the cellular uptake of PEGylated dextran nanogels. Nanoscopic particles showing

sustained release of a therapeutic molecule are possible candidates for treatment of ocular disorders, because repeated intravitreal injections can be avoided. Liposomes as drug delivery vehicles after intravitreal injections have been studied for two decades; they show significantly increased drug half-life and they minimize intraocular side-effects[50]. Hence we examined the intracellular behavior of the PEGylated lipid coated nanogels in retinal pigment epithelial (RPE) cells.

The first step in the (endosomal) uptake of these particles is binding to the cellular membrane. It is shown that the PEGylated particles bind to a lower extent to the cellular surface when compared to the non-PEGylated ones, both for the RPE as for the VERO cells. This arises from the negative charge of the cellular membrane, whereas PEGylation significantly lowers the (positive) surface charge of the dextran nanogels (as shown in Figure 5C). It is shown that the two types of nanogels are taken up by cells at 37°C (conditions under which endocytosis is performed) as fluorescent punctuations are observed in the cytosol of both types of cells, but no fluorescence was seen in the nucleus. This is probably due to the fact that the nucleopores are much smaller than these nanosized particles, thereby forming a barrier for the nanogels to enter the nucleus[51]. These results indicate that the presence of a PEG coated surface has no influence on the uptake and endosomal release of dextran nanogels by RPE and VERO cells, although the cellular binding of the PEGylated particles is somewhat lower. These results are in agreement with the findings of Vertut-Doï et al.[52], which show that the binding of EPC liposomes to the macrophage cell line J774 is higher than the same liposomes containing 5 mol% PEG_{2200}-CHOL. Surprisingly, the PEGylated liposomes do not differ so much from control liposomes with regard to endocytotic uptake.

As it is of great importance that the nanoparticles do not aggregate upon parenteral injection, since particles of several micrometers in diameter are able to clog in the lung capillaries[53] and submicron particles are rapidly cleared by the MPS[54]. For that purpose it is of great importance that the PEGylated nanogels are stable in human serum. It is however not evident to perform DLS measurements in serum, due to the abundance of small particles capable of light scattering. Therefore, the serum was first filtrated through a 2.0 µm filter. The nanogels did not show aggregation at ten-fold dilution with serum, however at 1:1 dilutions, aggregation was observed for both types of nanogel. So, despite the presence of a "protecting" PEGylated lipid layer, concentrated nanogel suspensions in serum aggregate after 7 hours. Nevertheless, these results are promising, since the injected nanogel dispersion would become very diluted in the bloodstream.

A critical issue in the *in vitro* and *in vivo* use of pH sensitive particles relates to the possibility that properties like stability, pH sensitivity and cell affinity may be changed as a consequence of their interactions with components of biological fluids or with cells. For that purpose PEG can be foreseen on the surface of particles showing pH dependent stability, forming a steric barrier around the nanogels. Particles entering the bloodstream are covered with opsonines very fast and as a result they are recognized by phagocytes and are removed within minutes. It is the

flexible nature of PEG which ensures that the particles are protected. When opsonins approach, the chains are compressed, resulting in opposing repulsive forces. To be effective, the PEG layer must be, however, thick enough. This generally implies that PEG chains have to be 2000 g/mol or more. Also the surface chain density and the conformation of the PEG molecules on the particles is very important. When the chain density is too low the PEG molecules take the typical mushroom configuration: the PEG chains are close at the surface and opsonins can freely bind to the particles' surface. At too dense occupancy the PEG chains lose their flexibility and exhibit the brush configuration. The optimal surface coverage is located somewhere in between the brush and mushroom configuration[7].

It has been reported that the acid catalyzed hydrolysis of the plasmalogen vinyl ether linkage in DPPlsC (Figure 3) triggers the calceine release from liposomes containing DPPlsC[32]. Also the use of lipids with high transition temperatures, like DPPC, have led to a significant decrease of leakage of the encapsulated drugs during circulation or in the extracellular milieu[55]. For that reason a DPPC:DPPlsC lipid coating in a 8:2 molar ratio containing 5 mol% DSPE-PEG_{2000} was hydrated with a 20% (w/w) dex-HEMA solution. It was shown both by DLS and visually that the pH sensitive lipid coating around the particle stays intact at physiological pH, but comes off at lower pH. This result is of great importance, since PEG often hampers endosomal escape.

CONCLUSION

The most common approach for enhancing the delivery of parenteral agents is through attachment of PEG moieties, referred to as PEGylation. By use of PEGylation the immunological, pharmacokinetic and hence pharmacodynamic properties of the drug delivery vehicle are often improved[56].

In this chapter we report on PEGylated lipid coated dextran nanogels. These are obtained by using PEGylated liposomes as a reaction mould for the polymerization of dex-HEMA. We reported on the easy and efficient production of PEGylated lipid coated nanogels. The particles have a lower surface charge resulting in lower binding properties to both VERO and RPE cells. Nevertheless, this is no obstacle for cellular uptake: PEGylated as well as non-PEGylated particles are taken up by these cells equally efficient. Both PEGylated and non PEGylated nanogels show serum stability, at sufficient dilution, Finally we have obtained pH responsive PEGylated particles where the lipid coating can be removed at lower pH values. This was achieved by the inclusion of a pH sensitive lipid, DPPlsC. This might offer possibilities as a dePEGylation strategy in endosomal compartments.

These particles show great promise for the intracellular delivery of therapeutic molecules with a cytosolic or nuclear target

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5

INFLUENCE OF FREE CHAINS ON THE SWELLING PRESSURE OF PEG-HEMA AND DEX-HEMA HYDROGELS

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Abstract

Self-exploding microgel particles show potential for advanced delivery of certain therapeutics. In order to be able to develop drug delivery systems that release their content based on the increase of osmotic pressure, insight in the osmotic behavior of the composing degrading hydrogels is of great importance. This study compares the degradation behavior of PEG-HEMA (hydroxyethyl methacrylated poly(ethylene glycol)) and dex-HEMA (hydroxyethyl methacrylated dextran) hydrogels. The degradation of PEG-HEMA gels takes several months to over a year, while that of dex-HEMA gels takes only days or weeks. The faster degradation kinetics of dex-HEMA networks can be attributed to stabilization of the keto-enol form by hydroxyl groups. Upon degradation of PEG-HEMA and dex-HEMA hydrogels respectively free PEG and free dextran chains are produced. We investigated the effect of unattached PEG and dextran chains on the swelling pressure of the degrading gels. It is found that low molecular weight free chains significantly increase the swelling pressure. However, the contribution of higher molecular weight free chains (MW > 10 kDa) is similar to that of the network chains.

INTRODUCTION

Recently biodegradable hydrogels have gained much attention because of their useful properties in various applications, such as drug release[1-8] and tissue engineering[9-13]. Previously our research group reported results on the synthesis and characterization of "self-rupturing microcapsules"[14-17] (Figure 1) consisting of a biodegradable dextran gel core surrounded by a (lipid or polymeric) membrane, which is permeable for small molecules (e.g., water and ions) but impermeable for the degradation products of the hydrogel (free polymer chains). In the course of the degradation process the swelling pressure of the hydrogel (π_{sw}) gradually increases[18]. Provided that the increase in swelling pressure is sufficient to overcome the rupture strength of the surrounding membrane, the latter ruptures (Figure 1D). The *time* of rupturing is primarily determined by the degradation kinetics of the gel core that governs the increase of π_{sw}.

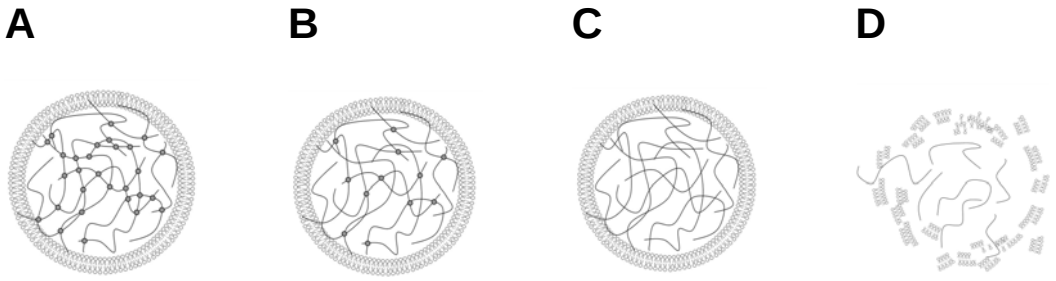

Figure 1. Schematic representation[18] of a "self-rupturing" microcapsule[14,15] based on a degradable microgel surrounded by a semipermeable membrane: (A) before degradation the polymer chains are connected into a three-dimensional network by chemical cross-links. (B) The gels described in this paper degrade by hydrolysis of the cross-links connecting the polymer chains. As degradation proceeds, the cross-link density decreases and free polymer chains are produced. (C) At the end of the degradation process the hydrogel becomes a polymer solution. (D) When the swelling pressure is sufficiently high the membrane will rupture.

To design self-rupturing dextran based microcapsules our research group recently investigated the swelling pressure of degrading hydroxyethyl methacrylated dextran (dex-HEMA, Figure 2A) hydrogels[18,19]. The polymer network in these hydrogels is formed by radical polymerization of a dex-HEMA solution. The HEMA based cross-links, which connect the dextran chains, contain hydrolysable carbonate esters that make the dex-HEMA hydrogels (bio)degradable (Figure 2B). The completely degraded dex-HEMA hydrogel forms a solution consisting of dextran and poly(HEMA) chains[16,17,20]

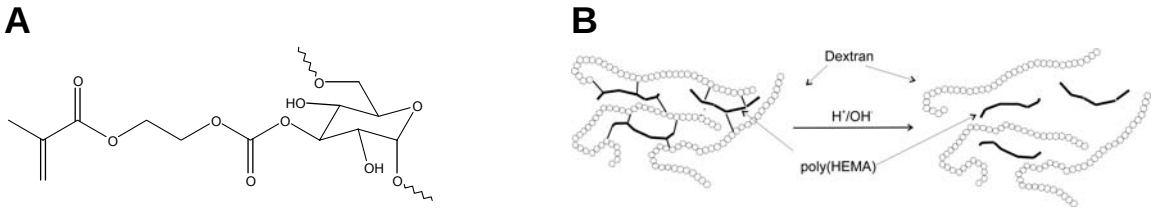

Figure 2. (A) Chemical structure of the monomer in dex-HEMA, i.e. glucopyranose substituted with HEMA and (B) schematic representation of a dex-HEMA network before and after degradation.

The swelling pressure of a neutral polymer gel is determined by two opposing effects[21]: the osmotic pressure (π_{osm}) that expands the network and the elastic pressure (π_{el}) that contracts the network:

$$\pi_{sw} = \pi_{osm} + \pi_{el} \qquad \text{eq. 1}$$

where π_{el} = –G', G' being the elastic (shear) modulus of the hydrogel. Equation 1 predicts that π_{sw} of a completely degraded hydrogel (i.e. G' = 0) is equal to the osmotic pressure of the solution of the degradation products. Stubbe et al. found previously that eq. 1 satisfactorily describes the osmotic pressure of dex-HEMA hydrogels in the course of degradation[18].

To break the membrane surrounding the microgel particles π_{osm} should exceed the tensile strength of the membrane. To satisfy this requirement polymers that exhibit high osmotic pressure in solution are ideal candidates.

In chapter 5 we report on the swelling pressure measurements for degrading hydroxyethyl methacrylated poly(ethylene glycol) (PEG-HEMA) gels (Figure 3A & B).

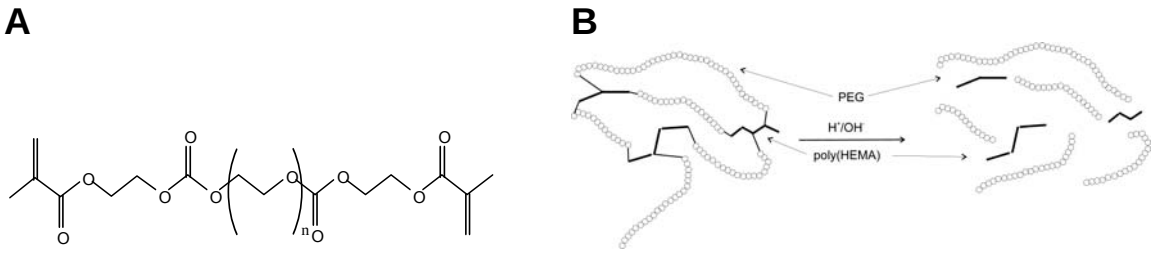

Figure 3. (A) Chemical structure of PEG-HEMA and (B) schematic representation of a PEG-HEMA network before and after degradation. The HEMA cross-links degrade through hydrolysis of the carbonate esters, resulting in PEG chains and poly(HEMA).

At identical cross-link density PEG-HEMA gels are expected to display higher π_{sw} than dex-HEMA gels since the osmotic pressure of the PEG solution exceeds that of the dextran solution at the same polymer concentration[22]. PEG is biocompatible and PEG based gels are widely used in drug delivery[9,23-27] and tissue engineering applications[9]. Rathi et al. studied thermoreversible hydrogels made of

the triblock copolymer PLGA-PEG-PLGA (PLGA being poly-DL-lactide-co-glycolide)[28,29]. Dissolving PLGA-PEG-PLGA in water results in a solution at room temperature that becomes a hydrogel at body temperature. Due to hydrolysis of the PLGA blocks, PLGA-PEG-PLGA hydrogels degrade slowly over a period of 4-6 weeks[28-30]. Other biodegradable PEG hydrogels have been made from PEG-fumarate and N,N'-methylene-bisacrylamide[31] or by conjugate addition of PEG multiacrylates (with a multi-armed structure) and dithiotreitol[27].

In this chapter we describe the synthesis of PEG-HEMA hydrogels and investigate their degradation behavior. To the authors' knowledge the osmotic properties of PEG-HEMA hydrogels have not been studied before. Our further aim is to determine the swelling pressure of degrading PEG-HEMA hydrogels at different stages of degradation. Particularly, we intend to quantify the effect of unattached PEG chains on the swelling pressure of degrading gels. To this end we made osmotic swelling pressure measurements on PEG-HEMA hydrogels containing controlled amounts of PEG chains of known molecular weights. We also compare the swelling and degradation behaviors of PEG-HEMA and dex-HEMA hydrogels.

EXPERIMENTAL SECTION

Materials

Dextran (from *Leuconostoc Mesenteroïdes*, M_n = 19 kDa), HEMA, dimethyl sulfoxide (DMSO < 0.01% water), $MgSO_4$, *N,N,N',N'*-tetramethylenediamine (TEMED), PEG (M_n = 200 Da; 600 Da and 1.5 kDa) and hydroquinone monomethyl ether (HQM) were purchased from Fluka Chemie AG. 4-(*N,N*-dimethylamino)pyridine (DMAP) and 1,1'-carbonyldiimidazole (CDI) were from Acros Chimica. PEG (M_n = 4 kDa; 10 kDa and 20 kDa), potassium persulfate (KPS) and dichloromethane (DCM) were purchased from Merck. Dextran (M_n = 600 Da and 800 Da) was obtained from Dextran Products. Dextran (M_n = 5 kDa and 77 kDa) were purchased from Sigma-Aldrich. Dialysis tubes with different molecular weight cut offs (MWCO, regenerated cellulose) were obtained from Spectrum Labs (The Netherlands).

Synthesis of PEG-HEMA and dex-HEMA

The synthesis of PEG-HEMA comprised of two steps: the first step being the activation of HEMA with CDI (resulting in HEMA-CI), the second step being the coupling of HEMA-CI to the terminal hydroxyl groups of PEG.

The synthesis of HEMA-CI was performed as described by van Dijk-Wolthuis et al.[17]. Briefly, 29 g (179 mmol) CDI was dissolved in 300 mL dichloromethane. 11.6 g (89 mmol) HEMA was added and the mixture was stirred for 1 h under N_2 at room temperature. Then the solution was washed with water to remove imidazole,

unreacted HEMA and CDI. The HEMA-CI solution in dichloromethane was dried over $MgSO_4$. Subsequently HQM was added to avoid the formation of poly(HEMA) and the solvent was evaporated resulting in a light yellow oil: HEMA-CI.

PEG-HEMA was synthesized as follows. 50 g (12.5 mmol) PEG (M_n = 4 kDa), dried in a vacuum oven for 24 h at room temperature, and 5 mg HQM were dissolved in 500 mL DMSO in a 1 L three-neck round bottomed flask under N_2 stream. After dissolving 10 g DMAP in the PEG/DMSO solution 14 g HEMA-CI (62.5 mmol, 5 eq.) was added dropwise while stirring vigorously. This mixture was allowed to react for 5 days under N_2. The reaction was terminated by decreasing the pH to 4.0 with HCl solution. DMSO was removed by dialysis against deionized water for one week (MWCO of the dialysis tubes was 2 kDa), the water was refreshed twice a day. Subsequently the solution was freeze dried and the fluffy white powder was stored at –20°C under N_2. Purity was determined by proton nuclear magnetic resonance spectroscopy (^{1}H NMR) in D_2O with a Gemini 300 spectrometer (Varian). The degree of methacrylation was determined by ^{1}H NMR and found to be 96%, i.e., 96% of the terminal hydroxyl groups of PEG were substituted with HEMA.

Importantly, in the storage of PEG and in the synthesis of PEG-HEMA the formation of PEG peroxides should be avoided[32]. Such peroxides spontaneously initiate the polymerization of PEG-HEMA solutions. To prevent peroxide formation we added HQM to the PEG-HEMA and stored the polymer at –20°C under N_2.

Dex-HEMA was prepared and characterized as described elsewhere[17]. Dextran (M_n = 19 kDa) was used in the synthesis of the dex-HEMA. The degree of substitution (DS), defined as the number of HEMA groups per 100 glucose units, was determined by ^{1}H NMR. The DS of the two dex-HEMA samples used in this study were 2.5 and 5.4, respectively.

Gelification procedure and swelling pressure measurements

The swelling pressure of dex-HEMA and PEG-HEMA hydrogels was measured using a home-made apparatus described elsewhere[18] and schematically represented. The device consists of two stainless steel chambers: a “sample chamber”, containing the hydrogel and the calibrated pressure transducer, and a “buffer chamber”. The chambers are separated by a semi-permeable membrane supported by a porous Bekipor frame, and further supported by a perforated Teflon cylinder. The nominal MWCO of the membrane was respectively 100 Da (for π_{sw} measurements on PEG-HEMA gels and on dex-HEMA gels containing 600 and 800 Da dextran chains) and 2 kDa (for π_{sw} measurements on dex-HEMA gels containing 5, 10, 19 and 77 kDa dextran chains).

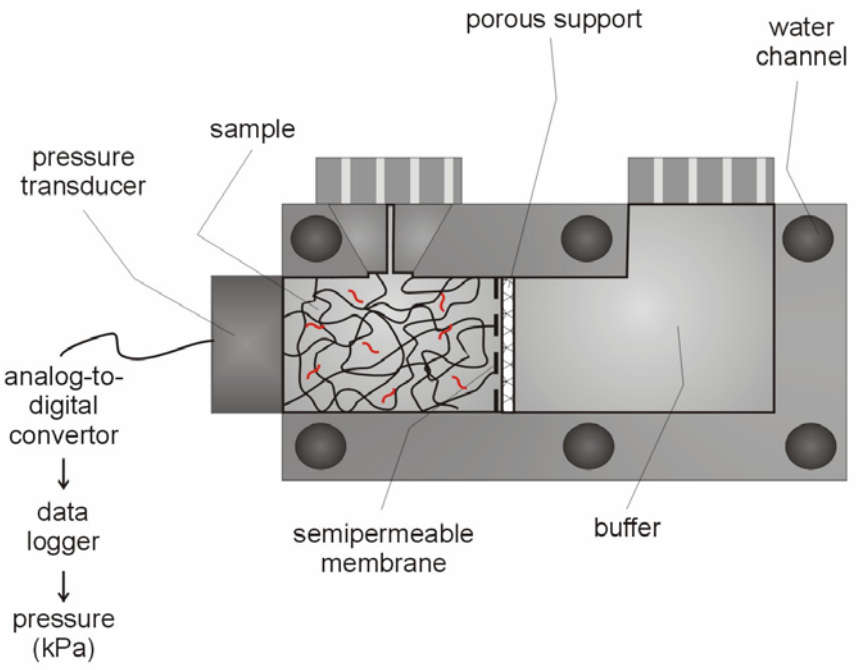

Figure 4. Schematic representation of the home-made osmometer. The hydrogel is moulded in the sample chamber resulting in perfect contact between pressure transducer and hydrogel and hydrogel and the semi-permeable membrane, which is supported on a porous wire frame. The water channels of the device are connected to a warm water bath allowing thermostatisation. The stainless steel pressure transducer is calibrated up to 690 kPa.

The *buffer chamber* was filled with phosphate buffer (PB, 10 mM, pH=7.5). The *sample chamber* was filled with dex-HEMA (or PEG-HEMA) hydrogels. To generate a hydrogel out of the polymer (dex-HEMA, PEG-HEMA) solutions KPS and TEMED were used as initiator and catalyst respectively, following the method as described by van Dijk – Wolthuis et al.[16].

To a deoxygenated dex-HEMA (or PEG-HEMA) solution in PB were added KPS solution (90 µL KPS solution/g gel; 0.05 g KPS/mL PB) and TEMED solution (50 µL TEMED solution/g gel; 20% (v/v) TEMED in deoxygenated PB, pH was adjusted to 8.5 with HCl). After homogenization one part of the solution was transferred into the sample chamber. The other part of the solution was poured into a cylindrical stainless steal mould (diameter 2.3 cm, height 2 mm); the gel slabs thus made in the mould were used to measure G'. The polymer volume fraction of the hydrogels was calculated using the relationship

$$\varphi = \frac{w_{pol}}{w_{pol} v_1 + \frac{w_{buffer}}{\rho}} v_1 \qquad \text{eq. 2}$$

where w_{pol} and w_{buffer} are respectively the weight of the polymer and the buffer, ρ is the density of the buffer (1.07 g/mL) and v_1 is the specific volume of the polymer (being 0.84 mL/g for PEG and 0.72 mL/g for dextran)[33].

As described above the PEG-HEMA solutions also contained KPS and TEMED. As KPS and TEMED cannot diffuse through the MWCO 100 Da membrane, we added the same amount of TEMED and KPS to the PB in the buffer chamber.

Rheological measurements

Rheological measurements on hydrogel slabs, prepared in the mould as described above, were made using an AR1000-N controlled stress rheometer (TA-instruments) according to a method described in detail by Meyvis et al.[34]. The elastic moduli of the gels were obtained from oscillation measurements at 1 Hz applying a constant strain of 0.5%. For G' measurements on degrading hydrogels, the hydrogel slabs were submerged in PB at 37°C. At regular time intervals, G' of the hydrogel slabs was measured.

Gel permeation chromatography

The molecular weights of the dextran samples used in this study were determined by gel permeation chromatography (Table 1). An aliquot (20 μl) of the samples (1% solution) was injected into a Waters GPC system (Waters 600 HPLC pump, Waters 2410 Refractive Index detector). A Plgel 5μm Mixed-C column (Polymer Laboratories) was used. The mobile phase was DMSO and the flow rate was 0.5 mL/min at 70°C. Pullulans with varying molecular weight (Polymer Laboratories) were used as molecular weight standards.

Determination of the critical overlap concentration

The critical overlap concentration (c*) of the PEG and dextran solutions was estimated from eq. 3[35]

$$c^* = \frac{1}{[\eta]} \qquad \text{eq. 3}$$

where [η] is the intrinsic viscosity

$$[\eta] = \lim_{c \to 0} \frac{\eta_{sp}}{c} = \lim_{c \to 0} \frac{\ln \eta_{rel}}{c} \qquad \text{eq. 4}$$

In eq. 4 η_{sp} is the specific viscosity, η_{rel} is the relative viscosity and c is the polymer concentration in g/100 mL. The kinematic viscosities were measured by a Micro-Ubbelohde viscometer with Lauda S5 detector, which was connected to Lauda PVS1 Processor Viscosity System with a PVS 2.46 analysis program. The measurements were performed at 4.0 $\pm$ 0.1°C. The flow time of the PEG and dextran solutions was at most 10% - 20% over the solvent flow time.

RESULTS AND DISCUSSION

Comparison between the osmotic pressure of PEG and dextran solutions

As outlined in the Introduction, the motivation of investigating the PEG-HEMA system is that totally degraded PEG-HEMA hydrogels are expected to exhibit a higher osmotic pressure than dex-HEMA hydrogels. Figure 2B and Figure 3B show that degradation of dex-HEMA and PEG-HEMA hydrogels produces dextran and PEG molecules, respectively. We made osmotic pressure measurements on solutions prepared from dextran and PEG samples of comparable molecular weights (approximately 20 kDa). Figure 5 shows that the osmotic pressure of the PEG solution is significantly higher than that of the dextran solution at identical polymer concentration. This result indicates that water is a thermodynamically better solvent for PEG than for dextran. Note that in the degradation process a small amount of poly(HEMA) chains is also produced (see Figure 2B and Figure 3B). However, its contribution to the osmotic pressure is negligible[18].

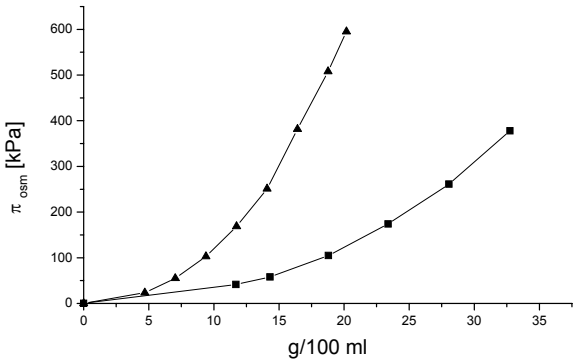

Figure 5. Osmotic pressure of dextran (M_n = 19 kDa ■) and PEG (M_n = 20 kDa ▲) solutions in water as measured in the swelling pressure device.

Degradation of PEG-HEMA hydrogels versus dex-HEMA hydrogels

Addition of KPS and TEMED to the PEG-HEMA (or dex-HEMA) solution results in gelation. In Figure 3B and Figure 2B are compared the structure of networks made from PEG-HEMA and dex-HEMA solutions: in PEG-HEMA the polymer molecules are end-linked, while in dex-HEMA the dextran side chains are cross-linked.

Figure 6A shows that the elastic modulus G' of PEG-HEMA hydrogels decreases in time when stored in buffer at 37°C. As expected G' also depends on the PEG-HEMA concentration, since with increasing PEG-HEMA concentration the cross-link density (which determines G') increases. The PEG-HEMA hydrogels degrade slowly. In a previous study Stubbe et al. found that the degradation of dex-HEMA hydrogels[19] is roughly ten times faster than that of PEG-HEMA hydrogels (compare Figure 6A and B). As illustrated in Figure 7, the faster degradation of dex-HEMA networks may be related to stabilization of the keto-enol form by hydroxyl groups[16].

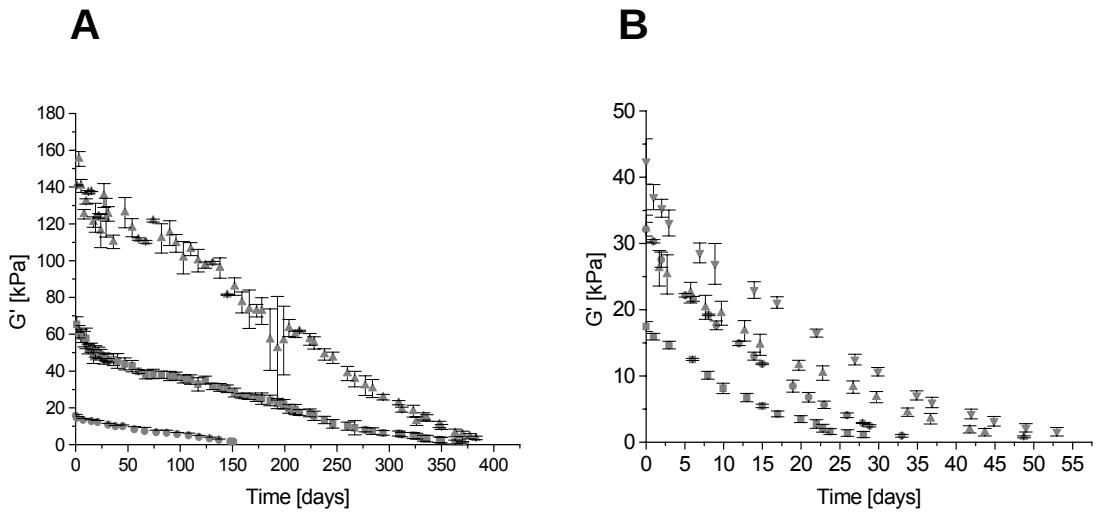

Figure 6. The elastic modulus of degrading PEG-HEMA. The PEG-HEMA concentration in the gels are 15% (•); 20% (■) and 25% (▲). (B) The elastic modulus of dex-HEMA hydrogels are as follows (Copyright ACS[18]): DS 7.5; 20% (▼);DS 5.0; 20% (▲); DS 2.5; 30% (•) and DS 2.5; 25% (■). The data are the average of three independent measurements.

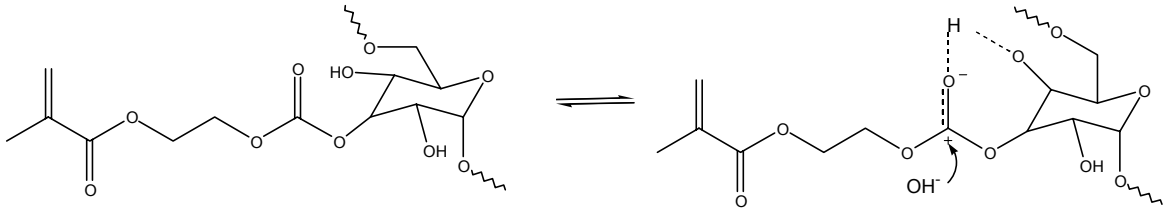

Figure 7. Keto-enol tautomerism in dex-HEMA: adjacent hydroxyl groups on the glucose unit facilitate keto-enol tautomerism.

Swelling pressure of PEG-HEMA hydrogels versus dex-HEMA hydrogels

Figure 6A shows that the complete degradation of PEG-HEMA gels takes several months. It is therefore difficult to measure the swelling pressure of degrading PEG-HEMA gels in *real-time*. Previously Stubbe et al. showed that the swelling pressure of dex-HEMA gels at various degradation times can be "mimicked" by gels containing known amounts of uncross-linked dextran (dex-HEMA/dextran gels)[18]. In this experiment the dextran chains were introduced into dex-HEMA solutions prior to cross-linking. Assuming that the osmotic behavior of degrading PEG-HEMA hydrogels can be mimicked by "PEG-HEMA/PEG" gels we determined the dependence of π_{sw} and π_{el} (= -G') on the concentration of the uncross-linked PEG in the gel. PEG-HEMA/PEG hydrogels were made by polymerization of PEG-HEMA solutions in the presence of known amounts of (vacuum dried) PEG.

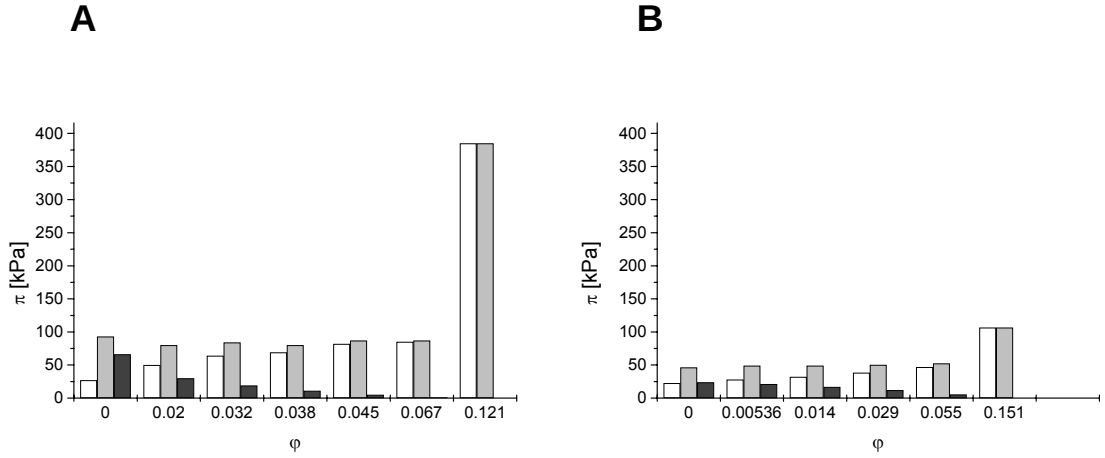

Figure 8. Swelling pressure (open), osmotic pressure (gray bars) and elastic pressure (black bars) of PEG-HEMA/PEG hydrogels (A) and dex-HEMA/dextran hydrogels (B; Copyright ACS[19]) containing increasing amounts of uncross-linked polymer chains (respectively dextran 19 kDa and PEG 4 kDa). The values on the x-axis indicate the volume fraction of the *uncross-linked* polymer in the corresponding gel. The total polymer volume fraction in the PEG-HEMA/PEG gels is 0.121. The total polymer volume fraction in the dex-HEMA/dex gels is 0.151.

Figure 8A shows that π_{sw} increases and π_{el} decreases with increasing PEG concentration. Remarkably, π_{osm} (= π_{sw} - π_{el}) only weakly depends on the PEG content at constant overall (cross-linked + uncross-linked) polymer concentration. However, when the gel turns into a polymer solution π_{osm} exhibits a jump-like increase. These results are consistent with similar observations made on dex-HEMA gels[18,36] (see Figure 8B).

The effect of uncross-linked chains on the osmotic properties of polymer gels has been studied on various systems. In a recent study on cross-linked DNA gels Horkay et al.[37] showed that π_{osm} of the gel is significantly lower than π_{osm} of the uncross-linked DNA solution. The lower π_{osm} of the gels was attributed to permanent elastic constrains generated by the cross-links. The present results suggest that in PEG-HEMA/PEG and dex-HEMA/dextran hydrogels the "free chains" strongly interact with the cross-linked polymer and behave like "cross-linked chains". When the gel is completely degraded, i.e., the polymer chains are no longer connected, the osmotic pressure increases and approaches that of the corresponding uncross-linked polymer solution.

Influence of the molecular weight of free chains in PEG-HEMA and dex-HEMA hydrogels on the osmotic pressure

To gain a better understanding of the contribution of free chains to the osmotic pressure we measured π_{osm} of PEG-HEMA/PEG and dex-HEMA/dextran hydrogels containing known amounts of uncross-linked polymer of different molecular weights. (The molecular weight of the dextran samples measured by GPC are listed in Table 1)

Table 1. Molecular weights of the dextran samples measured by GPC

Supplier info [kDa]	M_n [kDa]	M_w [kDa]	Pd
0.60	0.86	1.24	1.44
0.80	1.82	3.40	1.87
10.00	10.80	18.47	1.71
19.00	17.28	28.26	1.63
77.00	28.69	45.30	1.58

Figure 9A shows π_{sw}, π_{el} and π_{osm} of a dex-HEMA gel containing uncross-linked dextran chains of various molecular weights. For all the gels the volume fractions of cross-linked dex-HEMA and dextran were constant, $\varphi_{dex\text{-}HEMA}$ = 0.151 and $\varphi_{dextran}$ = 0.045 respectively. Clearly, π_{osm} of dex-HEMA/dextran gels containing 10, 19 and 77 kDa dextran chains is the same within the experimental error. Furthermore, π_{osm} is close to that of the corresponding dex-HEMA hydrogel in the

absence of uncross-linked dextran (compare Figure 8B and Figure 9A). However, the osmotic pressure of gels containing low molecular weight dextran oligomers (800 and 600 Da) is considerably higher. Similar results are shown in Figure 9B for a more densely cross-linked dex-HEMA/dextran network (DS = 5.4 (Figure 9B) and DS = 2.5 (Figure 9A)).

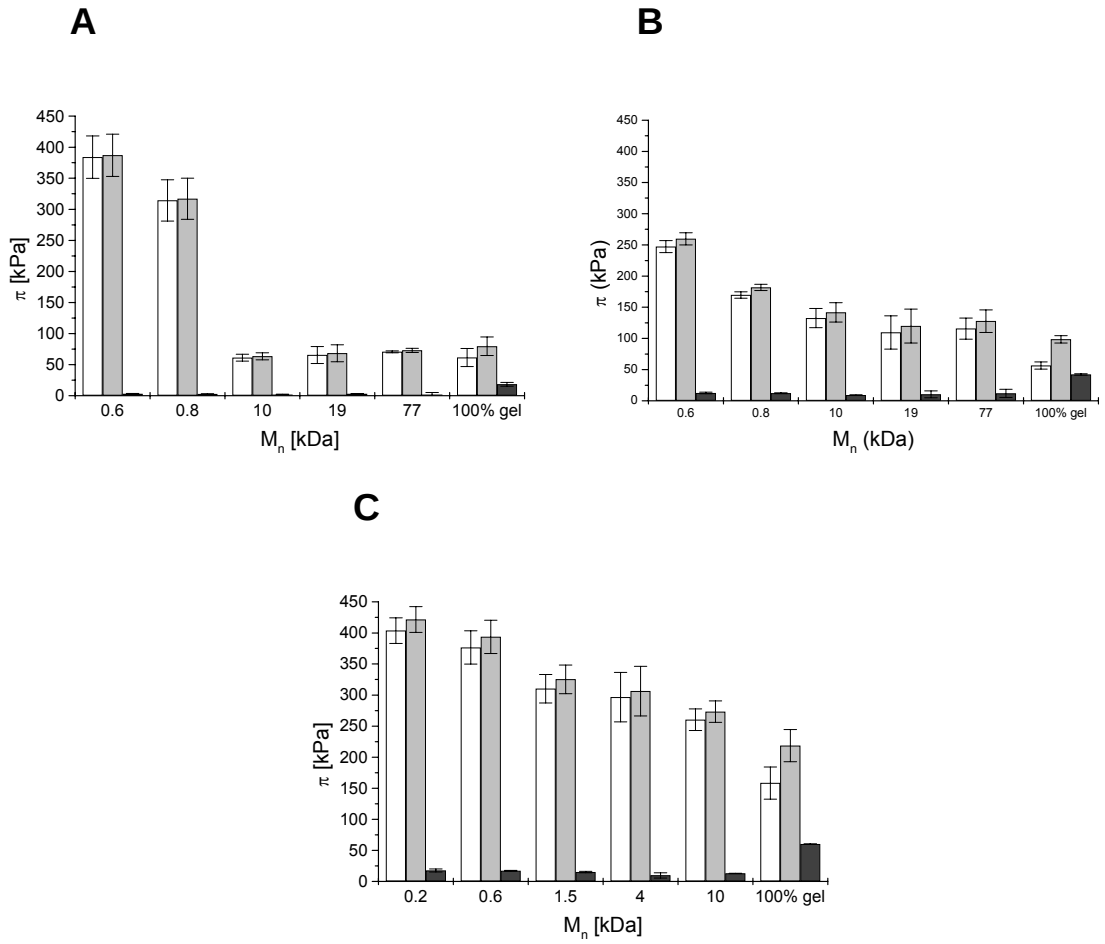

Figure 9. Swelling pressure (open), osmotic pressure (grey bars) and elastic pressure (black bars) of dex-HEMA/dextran hydrogels (A & B) and PEG-HEMA/PEG hydrogels (C). The DS of the dex-HEMA used in A is 2.5 while it is 5.4 in B. The x-axis indicates the molecular weight of the uncross-linked polymer. In all dex-HEMA/dextran gels (A, B) the total polymer volume fraction is 0.151, while the volume fraction of uncross-linked dextran is 0.045. In all PEG-HEMA/PEG gels (C) the total polymer volume fraction is 0.121, while the volume fraction of uncross-linked PEG chains is 0.036.

Figure 9C shows π_{sw}, π_{el} and π_{osm} of PEG-HEMA hydrogels containing free PEG chains of various molecular weights between 0.2 and 10 kDa. The results indicate that the osmotic pressure of the gels containing low molecular weight chains (0.2 and 0.6 kDa) is higher than that of the gels containing higher molecular weight (1.5; 4 and 10 kDa) free chains.

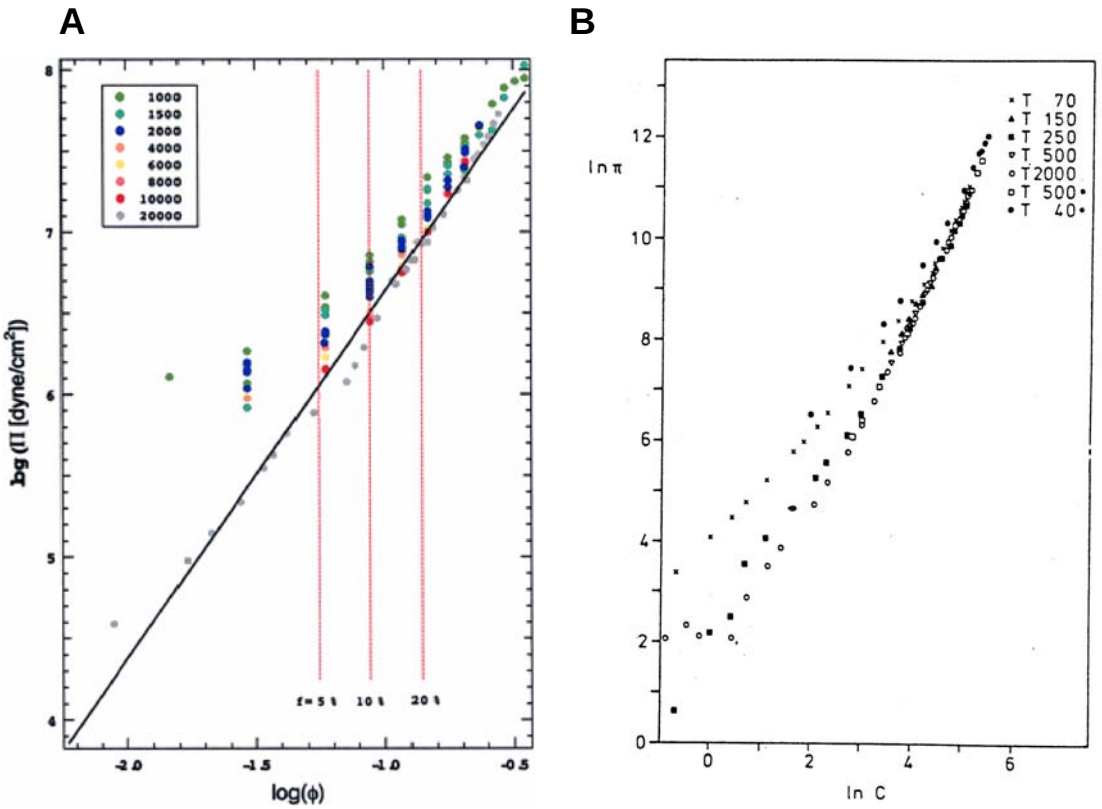

Figure 10. Dependence of the osmotic pressure on the polymer concentration for aqueous PEG (A) and dextran (B) solutions of different molecular weights. The data are obtained from Hansen et al.[38] for PEG and from Smit et al.[39] for dextran.

In general, the osmotic pressure of polymer solutions increases with the number of mobile chains. However, above the overlap concentration many polymer solutions exhibit universal behavior, i.e., the osmotic pressure is independent of the molecular weight of the polymer. This was indeed observed experimentally for PEG[38] and dextran[40] solutions (Figure 10A and B). In samples containing low molecular weight oligomers chemical effects may also be important. Different sites within a chain (chain ends and mid-chain segments) may interact differently with the cross-linked polymer. It is known that many water soluble polymers (e.g., poly(ethylene oxide, poly(acrylic acid)) form clusters due to association of the hydrophobic end groups[41-43]. The effect of end groups becomes less pronounced as the molecular weight of the polymer increases. We also note that in the present gel systems the concentration of the low molecular weight polymers was below their overlap concentrations (Figure 11)

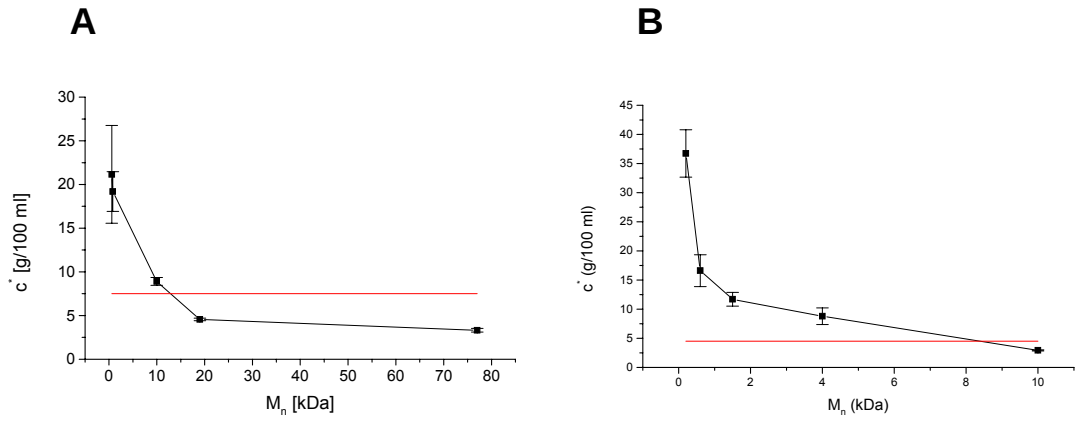

Figure 11: Overlap concentration c* as calculated from the intrinsic viscosity for dextran (A) and PEG (B) solutions as a function of the molecular weight. The horizontal line denotes the concentration of the uncross-linked dextran in the dex-HEMA/dextran hydrogels shown in Figure 9A and B, and the concentration of the uncross-linked PEG in the PEG-HEMA/PEG hydrogels shown in Figure 9C.

Clearly, low molecular weight free chains behave differently from the network chains. The difference between the osmotic contribution of the free chains and the network chains vanishes with increasing polymer molecular weight. Beyond a threshold molecular weight (for the PEG-HEMA/PEG system above 10 kDa) the free chains are practically indistinguishable from the network chains.

CONCLUSIONS

We demonstrated that degradable PEG-HEMA hydrogels can be made by radical polymerization of aqueous PEG-HEMA solutions using a simple two step synthesis route by coupling the carbonylimidazole activated HEMA to PEG. Total degradation of PEG-HEMA hydrogels (at pH 7.5) takes months to over a year, which is significantly longer than the degradation time (days to weeks) of dex-HEMA gels.

Upon degradation of PEG-HEMA and dex-HEMA hydrogels free PEG and dextran chains are produced. It is found that the osmotic pressure of the fully degraded PEG-HEMA gels exceeds that of similar dex-HEMA hydrogels. This observation is consistent with the results of osmotic pressure measurements made on solutions of the corresponding polymers.

To mimic partially degraded hydrogels we used PEG-HEMA gels containing known amounts of uncross-linked PEG and dextran chains. We studied the influence of the molecular weight of the uncross-linked polymers on π_{osm}. Different molecular weight dextran and PEG chains were incorporated into PEG-HEMA and dex-HEMA gels. We found that the difference between the contributions of network chains and free chains to the osmotic pressure decreases with increasing molecular

weight. Higher molecular weight free chains (MW > 10 kDa) behave like network chains.

The knowledge of the osmotic properties of degradable dex-HEMA and PEG-HEMA gels is of great significance for the design and optimization of self-rupturing microcapsules. The major application of the self-rupturing microcapsules (illustrated in Figure 1) is pulsed drug delivery. The ultimate goal is to insure that the drug molecules entrapped in the microcapsules are suddenly released when the surrounding membrane is ruptured. Clearly, to realize a sudden release the drug molecules should not leak through the membrane before the membrane becomes disrupted by the swelling pressure of the microgel. An increase in the swelling pressure due to degradation of the cross-linked polymer will exert a force on the membrane making it more permeable and leading to leakage of the drug. It is therefore important to use degradable microgels whose π_{osm} (and thus π_{sw}) remains low during the degradation process and suddenly increases when the gel turns into a polymer solution. Based on the results reported in the present chapter one may conclude that hydrogels made from high molecular weight polymers are favorable since the contribution of high molecular weight degradation products to the osmotic pressure is smaller.

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6

SCREENING POLY(ETHYLENE GLYCOL) MICRO- AND NANOGELS FOR DRUG DELIVERY PURPOSES

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Abstract

In chapter 5 we showed that poly(ethylene glycol) (PEG) is an ideal polymer for the synthesis of biodegradable hydrogels. This chapter investigates PEG based micro- and nanogels, with or without lipid coating, with the aim to slowly deliver encapsulated molecules. Hydroxyethyl methacrylated PEG (PEG-HEMA), PEG-HEMA with an oligo lactate spacer (PEG-lac-HEMA), and 8-armed PEG end capped with HEMA (✳-PEG-HEMA) were used. PEG-lac-HEMA matrices degraded very fast (in terms of days), while it took about one month for linear PEG-HEMA and several months for ✳-PEG-HEMA hydrogels to become degraded. PEG based microgels were made by use of an all aqueous technique and could be lipid-coated by mixing the microgels (made positively or negatively charged through copolymerization with respectively methacrylic acid and dimethyl aminoethyl methacrylate) with a suspension of oppositely charged liposomes. The release of fluorescently labeled molecules incorporated in the PEG based microgels could be clearly governed by the type of molecules used (lasting from hours to months). PEG based nanogels could be made using liposomes as a nanoscopic mold, resulting in particles with a PEG gel core surrounded by a lipid coating. BSA could be easily encapsulated in the PEG nanogels which released the BSA over a period of about one week.

INTRODUCTION

Micrometer and nanometer sized hydrogel particles show promise for the design of advanced drug delivery devices which may enhance the therapeutic index of many types of drugs. We aim to design "simple", tiny, biodegradable hydrogel particles which, after uptake by cells, slowly release their drug load in one or another cellular compartment or in the cytosol of the cells. We search for hydrogel particles which deliver the encapsulated drugs not only over a couple of hours, but also over days, weeks and even over months. Such slow delivering systems may be of interest for intracellular drug delivery in long living cells.

Previous research from the Hennink group showed that dextran-hydroxyethyl methacrylate (dex-HEMA) has great potential for the synthesis of dex-HEMA microgels[1-6]. In chapter 2 we showed that also dex-HEMA nanogels can be prepared using liposomes as a nanoscopic reactor. Hereby liposomes are filled with a dex-HEMA solution which is subsequently cross-linked with the formation of (lipid-coated) dex-HEMA nanogels[7,8]. Dex-HEMA micro- and nanogels are biocompatible[9] and degrade spontaneously under physiological conditions[10]. The degradation rate of the dex-HEMA micro- and nanogels depends on their cross-link density which is determined by the number of HEMA groups substituted on the dextran backbone[10].

In this chapter the focus is on biodegradable poly(ethylene glycol) (PEG) micro- and nanogels. PEG has been widely studied in pharmaceutical research for various purposes as (a) it is biocompatible[11,12], (b) (low molecular weight) PEGs are excreted by the body[13] and (c) PEG does not severely interact with blood- and cellular proteins[14]. Biodegradable PEG/poly(lactic acid) (PLA) and PEG/poly(glycolic acid) (PGA) block co-polymers have been investigated. When such co-polymers are end capped with acrylates they can be polymerized by UV or visible light. These macromers were polymerized in direct contact with tissues for the delivery of pDNA[15], growth factors[16], and vaccines[17]. For example, acrylate derivatized PLA-PEG-PLA is under investigation for the production of articular cartilage: after injecting acrylated PEG solutions together with chondrocytes or growth factors, transdermal photopolymerization leads to local formation of artificial cartilage[18,19]. Also PEG-diacrylates (without PLA or PGA blocks)[20] and polycaprolactone-*b*-poly(ethylene glycol)-*b*-polycaprolactone (PEG-CAP)[21] are developed for the *in situ* formation of articular cartilage. PEG hydrogels can also be formed without photopolymerization: in combination with glycolide and/or lactide oligomers PEG forms a hydrogel upon injection in a 37°C environment. Such PEG hydrogels have been reported to be non-cytotoxic[22] and biodegradable under physiological conditions. They are under investigation for the controlled delivery of insuline[23,24], pDNA[25] and other hydrophilic or lipophilic drugs (for example ketoprofen[26], spironolactone[26], misoprostol[25], 5-fluorouracil[27], indomethacin[27] and paclitaxel[28]).

PEG based hydrogels which are responsive to various stimuli have been reported as well. Andreopoulos et al. developed photoresponsive PEG based hydrogel membranes which can be cross-linked upon exposure to >300 nm light and

photodegrade when exposed to UV light (254 nm)[29,30]. By the co-polymerization of poly(N-isopropylacrylamide), temperature responsive PEG based nanogels[31], microgels[32] and macroscopic matrices[33] have been reported as well. Very recently PEG based "cell receptor responsive hydrogels" were described. Hydrogels were prepared by the interaction of multi-armed heparin modified PEG and the vascular endothelial growth factor (VEGF), a heparin binding growth factor[34]. In the presence of VEGF receptors, on the surface of VEGF receptor expressing cells, VEGF is exchanged between the hydrogel and the VEGF receptor, resulting in the dissolution of the hydrogel and the release of VEGF to the cell. It was shown that such type of hydrogels increase proliferation of VEGF responsive cell lines.

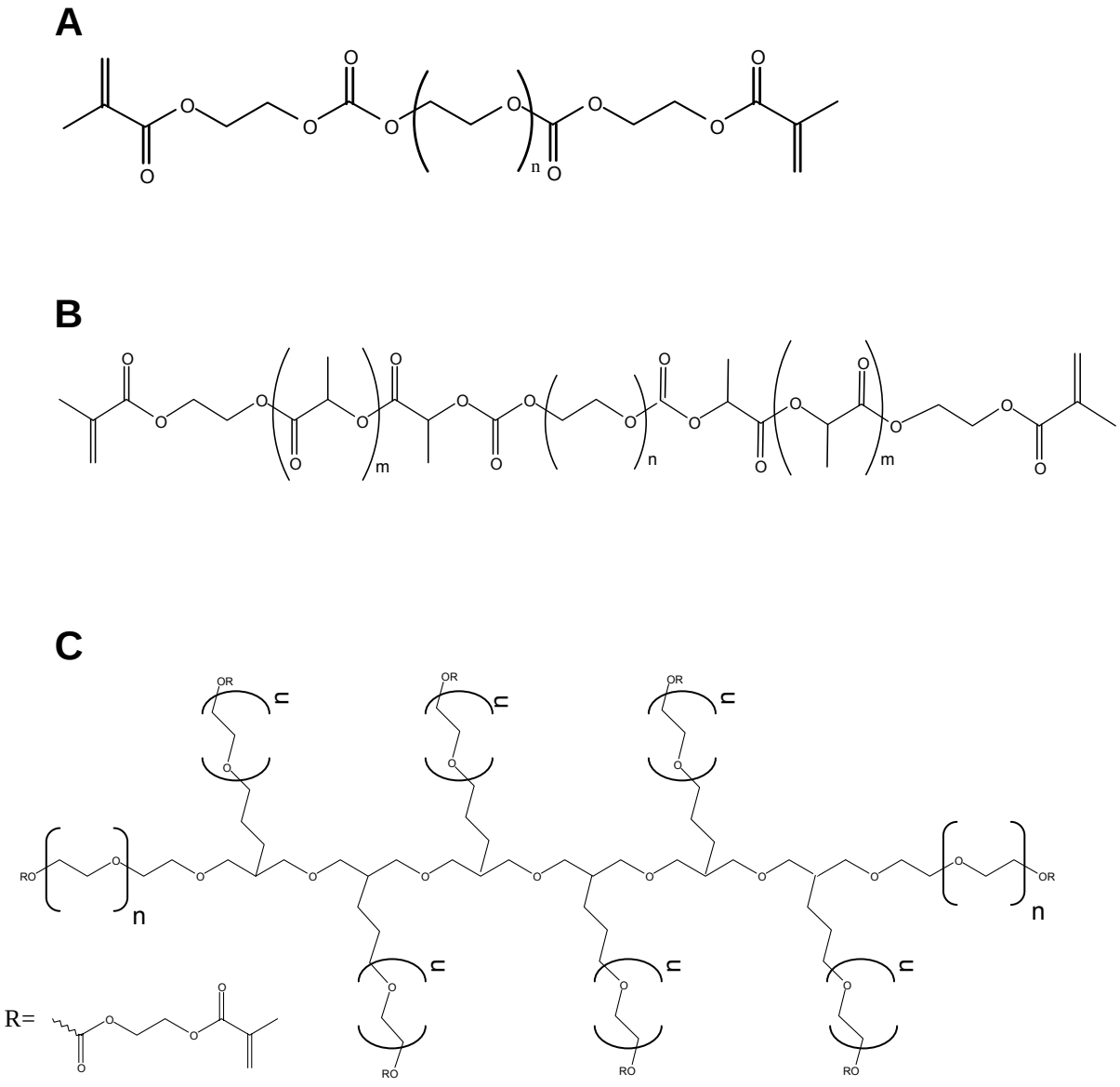

Figure 1. Chemical structure of the monomer in PEG-HEMA (A), PEG-lac-HEMA (B) and ✳-PEG-HEMA (C).

The major part of previous research on PEG hydrogels concerned the synthesis, physicochemical and biological evaluation of *macroscopic* hydrogel slabs. The present study, in which PEG-HEMA (Figure 1A), star shaped PEG-HEMA (Figure 1B) and PEG-lactate-HEMA (Figure 1C) were used, deals with the following questions. (a) Is it possible to make PEG based *micron*- and *nanosized* hydrogel particles? (b) Can we substantially vary the degradation time of PEG micro- and nanogels with the aim to tailor the release profiles of entrapped molecules? (c) How to surround PEG micro- and nanogels with a lipid coating as this may be of interest to modify their release characteristics and to optimize their cellular uptake and intracellular fate.

EXPERIMENTAL SECTION

Materials

$MgSO_4$, *N,N,N',N'*-tetramethylenediamine (TEMED), dimethyl aminoethyl methacrylate (DMAEMA) and hydroquinone monomethyl ether (HQM) were purchased from Fluka Chemie AG (Switzerland). 4-(*N,N*-dimethylamino)pyridine (DMAP) and 1,1'-carbonyldiimidazole (CDI) were from Acros Chimica (Belgium). Bovine serum albumin was purchased from Sigma Aldrich (Germany). Lactide was purchased from Purac Biochem (The Netherlands). Poly(ethylene glycol) (PEG, M_n = 4 kDa; 10 kDa and 20 kDa), potassium persulfate (KPS) and Triton X 100 (TX 100) were purchased from Merck (Germany). Star shaped PEG (✳-PEG, 8 arms, 10 kDa) and fluorescein-isothiocyanate-PEG (FITC-PEG) were obtained from Nektar (Japan). Dextran with a molecular weight of 40 kDa (dexT40) was obtained from Amersham Bioscience (Sweden). Dialysis tubes with different molecular weight cut offs (MWCO, regenerated cellulose) were obtained from Spectrum Labs (The Netherlands). Irgacure 2959 (I2959) was a free sample from Ciba Specialty Chemicals (Belgium). SOPC, DOTAP, CHOL, DPPC, DPPG and rho-DOPE (respectively being 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine;1,2-dioleoyl-3-trimethylammonium propane chloride; cholesterol, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; 1,2-dipalmitoyl-*sn*-Glycero-3-[phospho-*rac*-(1-glycerol)] (Sodium Salt) and rhodamine-dioleoyl-phosphatidylethanolamine) were purchased from Avanti Polar Lipids (Alabaster, USA).

Synthesis of PEG-HEMA and ✳-PEG-HEMA

For the synthesis of PEG-HEMA (Figure 1A) we refer to the previous chapter[35]. The synthesis of ✳-PEG-HEMA (Figure 1B) is very similar to the synthesis of PEG-HEMA. In a first step HEMA-CI is synthesized as described before[35]. The second step, being the coupling of HEMA-CI to the terminal hydroxyl groups of PEG, occurs as follows. ✳-PEG (M_n = 10 kDa), dried in a vacuum oven for 24 h at room temperature, and 5 mg HQM are dissolved in 10 mL DMSO in a 25 mL

three-neck round bottomed flask under N_2 stream. After dissolving 0.2 g DMAP in the ✳-PEG/DMSO solution, HEMA-CI is added drop wise while stirring vigorously. The amount of added HEMA-CI depends on how many arms of ✳-PEG have to become substituted with HEMA and is calculated as follows (eq. 1):

$$m_{HEMA-CI} = \frac{m_{*-PEG} \times n}{MW_{*-PEG}} \times MW_{HEMA-CI} \times 1{,}5 \qquad \text{eq. 1}$$

in which $m_{HEMA-CI}$ stands for the mass of HEMA-CI to be added, m_{*-PEG} is the mass ✳-PEG in the solution, n is the desired number of PEG-arms to be substituted by HEMA, MW_{*-PEG} and $MW_{HEMA-CI}$ are the molecular weight of respectively ✳-PEG (10 kDa) and HEMA-CI (224 Da). A factor 1,5 is taken into account as the incorporation efficiency of the acrylate groups on the terminal alcohol group on ✳-PEG is not 100%.

This mixture was allowed to react for 5 days under N_2. The reaction was terminated by decreasing the pH to 4.0 with HCl. DMSO was removed by dialysis against deionized water for one week (MWCO of the dialysis tubes was 2 kDa), the water was refreshed twice a day. Subsequently the solution was freeze dried and the fluffy white powder was stored at –20°C under N_2. Purity was determined by ^{1}H NMR in D_2O with a Gemini 300 spectrometer (Varian). The degree of substitution of the terminal alcohol groups on PEG with acrylate was determined using ^{1}H NMR from the ratio of the integral value of the acrylate (~5.8 or 6.4 ppm) to the PEG star (~3.6 ppm). The degree of substitution (DS; i.e. the extent of acrylation) was calculated by equation 2:

$$DS = \frac{I_{CH}}{I_{CH_2}} \times \frac{4 \times M_n}{MW_{EO}} \qquad \text{eq. 2}$$

in which I_{CH} and I_{CH2} are the vinylic integral and the oxyethylene integral, respectively. MW_{EO} is the molecular weight of the monomer in PEG (i.e. ethylene oxide), being 44 Da. The factor 4 represents the number of protons in the ethylene oxide molecule.

Importantly, in the storage of (✳-)PEG and in the synthesis of (✳-)PEG-HEMA the formation of PEG peroxides should be avoided[36] as peroxides would spontaneously initiate the polymerization of (✳-)PEG-HEMA solutions. To prevent peroxide formation we added HQM to the (✳-)PEG-HEMA and stored the polymer at –20°C under N_2.

Synthesis of PEG-lac-HEMA

The synthesis of PEG-lac-HEMA (Figure 1C) occurred in three steps. First, L-lactide was grafted onto HEMA in the presence of stannous octoate ($SnOct_2$), yielding HEMA-lactate. Second, HEMA-lactate was activated with CDI resulting in HEMA-lactate-CI. Third, HEMA-lactate-CI was coupled to PEG, to yield PEG-lactate-HEMA (PEG-lac-HEMA).

The synthesis of HEMA-lactate and HEMA-lactate-CI has been described in detail by van Dijk-Wolthuis et al.[37]. HEMA-lactate consisting of 2 lactyl residues was synthesized. The grafting of HEMA-lactate-CI to PEG occurred as follows. 50 g (12.5 mmol) PEG (M_n = 4 kDa), dried in a vacuum oven for 24 h at room temperature, and a spatula tip of HQM were dissolved in 500 mL DMSO in a 1 L three-neck round bottomed flask under N_2 stream. After dissolving 10 g DMAP in the PEG/DMSO solution 23 g HEMA-lactate-CI (62.5 mmol, 5 eq.) was added drop wise while stirring vigorously. This mixture was allowed to react for 4 days under N_2. The workup procedure was performed as described above for PEG-HEMA and ✳-PEG-HEMA.

Rheological measurements on degrading PEG based hydrogel slabs

PEG-HEMA, PEG-lac-HEMA and ✳-PEG-HEMA solutions were polymerized by the use of KPS (0.05 g KPS/mL in PB solution) and TEMED (20% (v/v) TEMED solution in deoxygenated phosphate buffer (PB), pH was adjusted to 8.5 with HCl) following the method as described by van Dijk-Wolthuis et al.[38]. Briefly, 50 µL of TEMED solution was added to 1 g of polymer solution. After homogenization, 90 µL of KPS solution was added to the system to initiate gelation.

The PEG-HEMA, PEG-lac-HEMA and ✳-PEG-HEMA hydrogel slabs, prepared in cylindrical moulds with a diameter of 2.3 cm and a height of 2 mm, were rheologically characterized using an AR1000- N controlled stress rheometer (TA-instruments) according to a method described in detail by Meyvis et al.[39]. For G' measurements on degrading hydrogels, the hydrogel slabs were submerged in PB at 37°C. At regular time intervals, G' of the hydrogel slabs was measured.

Synthesis of PEG-HEMA, ✳-PEG-HEMA and PEG-lac-HEMA microgels

PEG-HEMA, ✳-PEG-HEMA and PEG-lac-HEMA microgels were prepared as follows[4]: Briefly, a deoxygenated aqueous solution of methacrylated PEG (0.25 g of a 30% (w/w) solution in PB) and a dextran solution (5 g of a 40% (w/w) solution dexT40 in PB) were mixed with each other. The mixture was vigorously mixed with a vortex for 1 min under a N_2 atmosphere to obtain a water-in-water emulsion. The resulting emulsion was allowed to stabilize for 10 min. Subsequently 100 µL TEMED solution and 180 µL KPS solution (composition as described above) were added to

the emulsion. After gentle mixing the emulsion was incubated without stirring for 30 min at 37°C to cross-link the methacrylate groups in the PEG chains. The cross-linked microgels were purified by 3 washing steps with 10 mL PB. The microgels were collected by centrifugation and resuspended in 5 mL PB.

To prepare negatively and positively charged dex-HEMA microgels respectively methacrylic acid (30 µL MAA, Figure 2A) or dimethyl aminoethyl methacrylate (30 µL DMAEMA, Figure 2B) were added to the starting methacrylated PEG solution. To prepare fluorescently labeled methacrylated PEG microgels, 40 µL of a FITC-PEG solution (25 mg/mL in PB) was added to the methacrylated PEG solution.

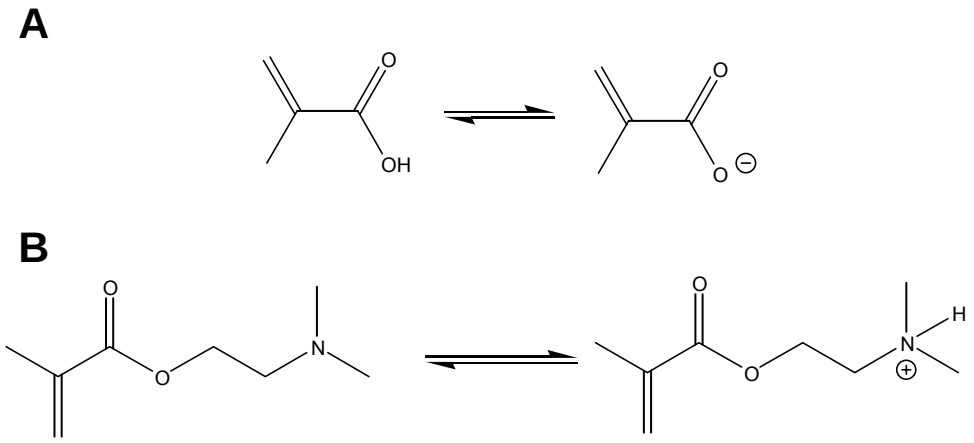

Figure 2. Chemical structure of MAA (A) and DMAEMA (B).

Lipid coating of (✳-)PEG-HEMA and PEG-lac-HEMA microgels

The methacrylated PEG microgels were coated with a lipid membrane by the use of liposomes. The liposomes were prepared by dissolving 5 mg of the desired lipids (respectively DOPC:DOTAP in a 9:1 molar ratio and DPPC:DPPG:CHOL in a 4:1:5 molar ratio) in 200 µL chloroform. 0.05 mol% of rho-DOPE was added to make the lipid film fluorescent. The chloroform was removed by use of N_2 while gently spinning the test-tube, leaving a thin lipid film on the bottom of the tube. The remaining chloroform was removed from the lipid film by vacuum drying during for at least 4h. The liposome dispersion was obtained by hydrating the lipid film with 1 mL PB (at a temperature at least 10°C above the phase transition temperature during 30 min and vortexing every 5 min). For the DOPC:DOTAP liposomes this was at 25°C, for the DPPC:DPPG:CHOL liposomes this was 45°C.

To coat the methacrylated PEG microgels, to a 1 mL (negatively or positively charged) liposome dispersion 2 mL of an (oppositely charged) microgel dispersion was added and incubated for 20 min at 25°C to allow sorption of the liposomes to the surface of the microgels. The excess of lipid was removed by density gradient centrifugation.

Confocal laser scanning microscopy

Confocal laser scanning images were recorded with a Bio-Rad MRC 1024 microscope using a water immersion objective lens (Plan Apo 60X, NA 1.2, collar rim correction, Nikon) and a krypton/argon laser.

Release of FITC-PEG from degrading (✳-)PEG-HEMA and PEG-lac-HEMA microgels

10 mL of a microgel dispersion was incubated at 37°C and mechanically agitated. At regular time intervals the dispersion was centrifuged (3 min at 100×g) and 2 mL of the supernatant was withdrawn and replaced by 2 mL of PB. The supernatant samples were stored at 4°C until further analysis.

The fluorescence intensity of the supernatant samples was measured with a Wallac Victor 2 (Perkin Elmer) plate reader. The fluorescence values were normalized against the fluorescence values measured at the end of the release experiments. It was verified that the measured fluorescence values belonged to the range in which a linear relation exists between the concentration of the FITC-PEG solutions and their fluorescence.

Preparation of (protein loaded) PEG-HEMA nanogels

PEG-HEMA nanogels were prepared using liposomes as a nanoscaled reactor, as described for dex-HEMA nanogels in chapter 2[7]. A conventional procedure to prepare the liposomes was used. A 5 mg lipid film of SOPC was made as described above. This dry lipid film was hydrated with 1 mL of a PEG-HEMA solution (i.e. 20% (w/w) PEG-HEMA in 50 mM PB at pH 7.0) which contained 0.05% I2959 as a photo initiator. The resulting dispersion was placed at 25°C for 30 minutes while vortexing every 5 minutes. This dispersion of large vesicles was aged overnight. Next, the dispersion was extruded with a LiposoFast Pneumatic-Actuator (Avestin) provided with a 400 nm polycarbonate membrane (Whatman International). After 11 back-and-forth passages of the dispersion through the extrusion membrane, the liposome dispersion was diluted 10 times with PB. This dilution was necessary as not all the PEG-HEMA was entrapped in the liposomes. If not diluted this “free” PEG-HEMA would form a gel in the polymerization step, thereby enclosing the liposomes in a polymer matrix. The PEG-HEMA containing liposomes were subsequently exposed to UV light (365 nm from a Bluepoint 2.1 UV source, Honle UV Technology) at 25°C for 450 s which cross-linked the PEG-HEMA solution in the liposomes with the formation of “lipid-coated PEG-HEMA nanogels”. To obtain “naked PEG-HEMA nanogels” the lipid layer was removed by adding 20.0 µL of a 100 mM solution of TX 100 to 1 mL of the lipid-coated nanogel dispersion.

To prepare bovine serum albumin (BSA) loaded PEG-HEMA nanogels, BSA (50 mg/mL) was added to the PEG-HEMA solution used to hydrate the dry lipid film.

Dynamic light scattering (DLS) analysis on degrading PEG-HEMA nanogels

To study the degradation of the nanogels, a cuvette was filled with 1.2 mL of the nanogel dispersions and sealed with Parafilm™ to avoid contamination with dust particles. The cuvette was placed at 37°C and the size distribution of the degrading particles was determined by dynamic light scattering (Autosizer 4700, Malvern Instruments) at regular times.

Protein release from degrading PEG nanogels

The amount of proteins released from the degrading PEG nanogels was measured as described in chapter 3.[8]. A Vivaspin centrifugation filtration device (having a membrane with a 300 kDa MWCO, Vivascience) was filled with a known weight (at least 2,5 g) of a dispersion of BSA loaded PEG nanogels in PB. Respectively before the nanogels began to degrade and at different times during their degradation (which occurred at 37°C), the amount of released BSA was determined by centrifugation of the dispersion for 3 minutes (at 100×g). The filtrate was stored (at 4°C) for BSA analysis and PB was added to the nanogel dispersion (remaining in the Vivaspin device) until the original weight of the dispersion. The release of the BSA was measured until the nanogels were completely degraded, as could be detected by DLS measurements.

The protein concentration in the samples was measured by Reversed Phase High Performance Liquid Chromatography (RP-HPLC, LaChrom Elite). An aliquot (2 μl) of the samples was automatically injected into a RP-HPLC system (Hitachi LaChrom Elite, L-2100 SMASH pump, L-2200 autosampler, L-2300 column oven and L-2450 Diode Array Detector). A RP C4 column (Alltech, 300Å, 5 μm, 25 cm) was used. A water/acetonitrile mixture (the solvent's composition and the concentration gradient in function of time are given in chapter 3), adjusted to pH 2.0 with trifluoroacetic acid (TFA, Sigma) was used as the mobile phase at a constant flow rate of 0.75 mL/min. A calibration curve was obtained by injecting various volumes of a 5 mg/mL protein solution in PB. The protein concentration in the samples was calculated from the area under the BSA peak, using the calibration curve.

RESULTS AND DISCUSSION

Degradation behavior of PEG-HEMA, PEG-lac-HEMA en ✳-PEG-HEMA gels

Figure 3A-C shows the elastic moduli of PEG-HEMA, PEG-lac-HEMA and ✳-PEG-HEMA hydrogels degrading in phosphate buffer (pH 7.0) at 37°C. Figure 3A shows that it takes approximately 6 months to degrade a PEG-HEMA (15% (w/w)) hydrogel while PEG-lac-HEMA hydrogels (even those which contain 65% (w/w) PEG-lac-HEMA) are already degraded after some days (Figure 3B). It suggests that the

lactate esters in PEG-lac-HEMA hydrolyze more rapidly than the carbonate esters in PEG-HEMA. This is in agreement with observations by Van Dijk-Wolthuizen et al.[10] who showed that dex-HEMA degraded slower compared to dex-lac-HEMA. Figure 3C shows that the degradation of ✳-PEG-HEMA hydrogels proceeds over several months. As ✳-PEG-HEMA molecules have multiple methacrylate groups per molecule (note that a linear PEG-HEMA chain only bears two methacrylate groups), the concentration of cross-links in ✳-PEG-HEMA hydrogels is much higher than in linear PEG-HEMA hydrogels with the same PEG concentration. Subsequently, to dissolve the network more cross-links have to be hydrolyzed which takes longer times.

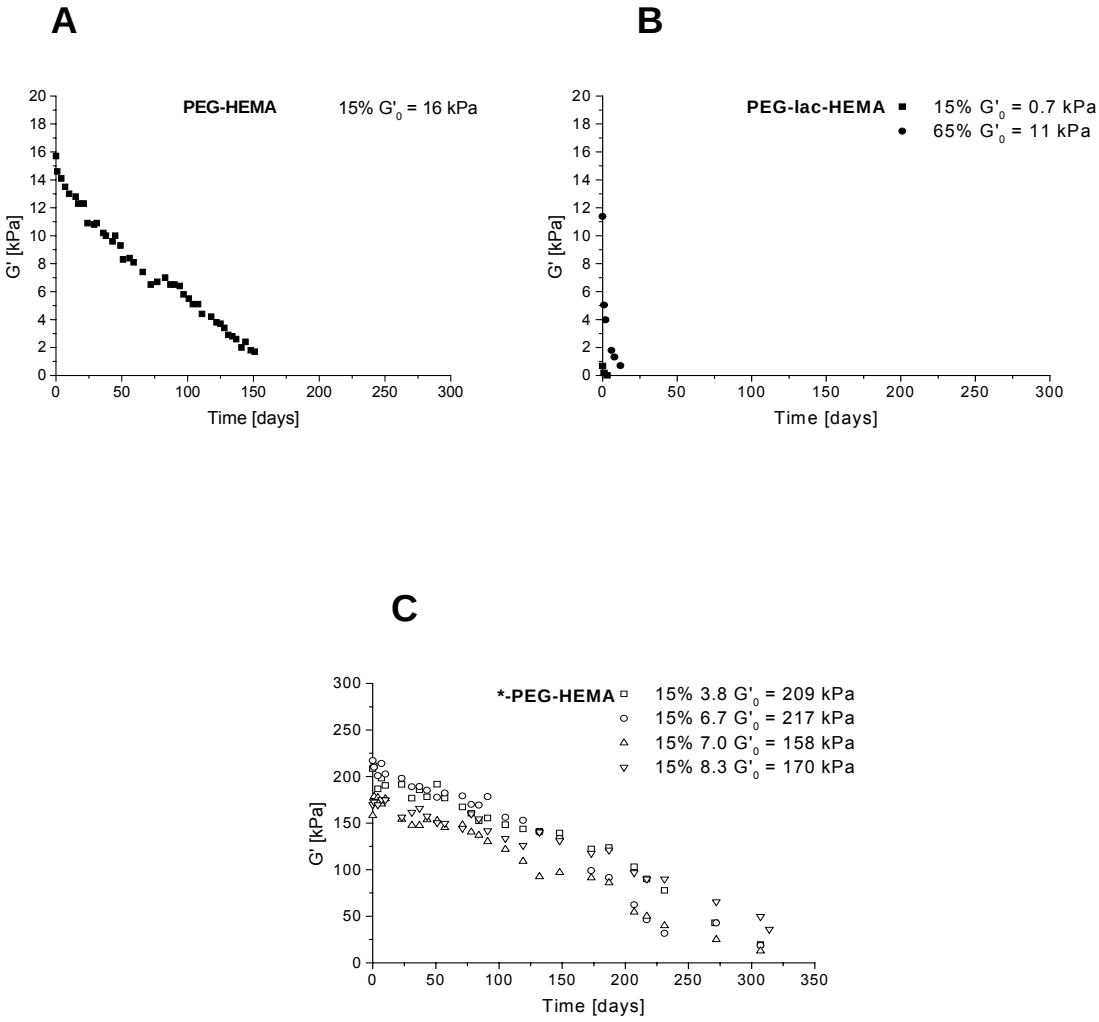

Figure 3. Elastic moduli of degrading PEG based hydrogels. (A) PEG-HEMA hydrogels. (B) PEG-lac-HEMA hydrogels. (C) ✳-PEG-HEMA hydrogels. The number of arms substituted with HEMA (per ✳-PEG) is respectively 3.8, 6.7, 7.0 and 8.3. G0 is the elastic modulus of the gel before degradation.

Obviously, as can be seen in Figure 3C, increasing the DS from 3.8 till 6.7 of ✳-PEG-HEMA hydrogels (while keeping the ✳-PEG-HEMA concentration constant) results in a network with a higher cross-link density and thus a higher G_0 (i.e. the initial G' value of the hydrogels before degradation). However, a further increase in DS (to 7.0 and 8.3) results in softer gels which we attribute to higher degree of intramolecular cross-linking. *Intra*molecular cross-links – in contrast to *inter*molecular cross-links- do not contribute to the network's elasticity[40].

PEG based microgels

Synthesis of PEG-HEMA, PEG-lac-HEMA and ✳-PEG-HEMA microgels. Since aqueous solutions of PEG-HEMA and dextran are immiscible, a PEG-HEMA/dextran emulsion with dextran as continuous phase, can be made[2]. When KPS and TEMED are added to PEG-HEMA/dextran emulsions, the methacrylated PEG droplets are expected to polymerize yielding PEG based microgels. The CLSM images in Figure 4(A_1, B_1 and C_1) show that polymerization of the emulsification indeed results in PEG-HEMA, PEG-lac-HEMA and ✳-PEG-HEMA microgels.

To make charged PEG-microgels we co-polymerized respectively MAA and DMAEMA with PEG-HEMA, as this results in negatively and positively charged particles respectively[1]. Figure 4A_2, B_2 and C_2 shows CLSM images of respectively PEG-HEMA-MAA, PEG-lac-HEMA-MAA and ✳-PEG-HEMA-MAA microgels. While also positively charged PEG-HEMA-DMAEMA and ✳-PEG-HEMA-DMAEMA could be easily produced (Figure 4A_3 and C_3), it seemed hard to obtain PEG-lac-HEMA-DMAEMA microspheres which might be attributed to the fact that PEG-lac-HEMA-DMAEMA degrades too fast to make stable microgels.

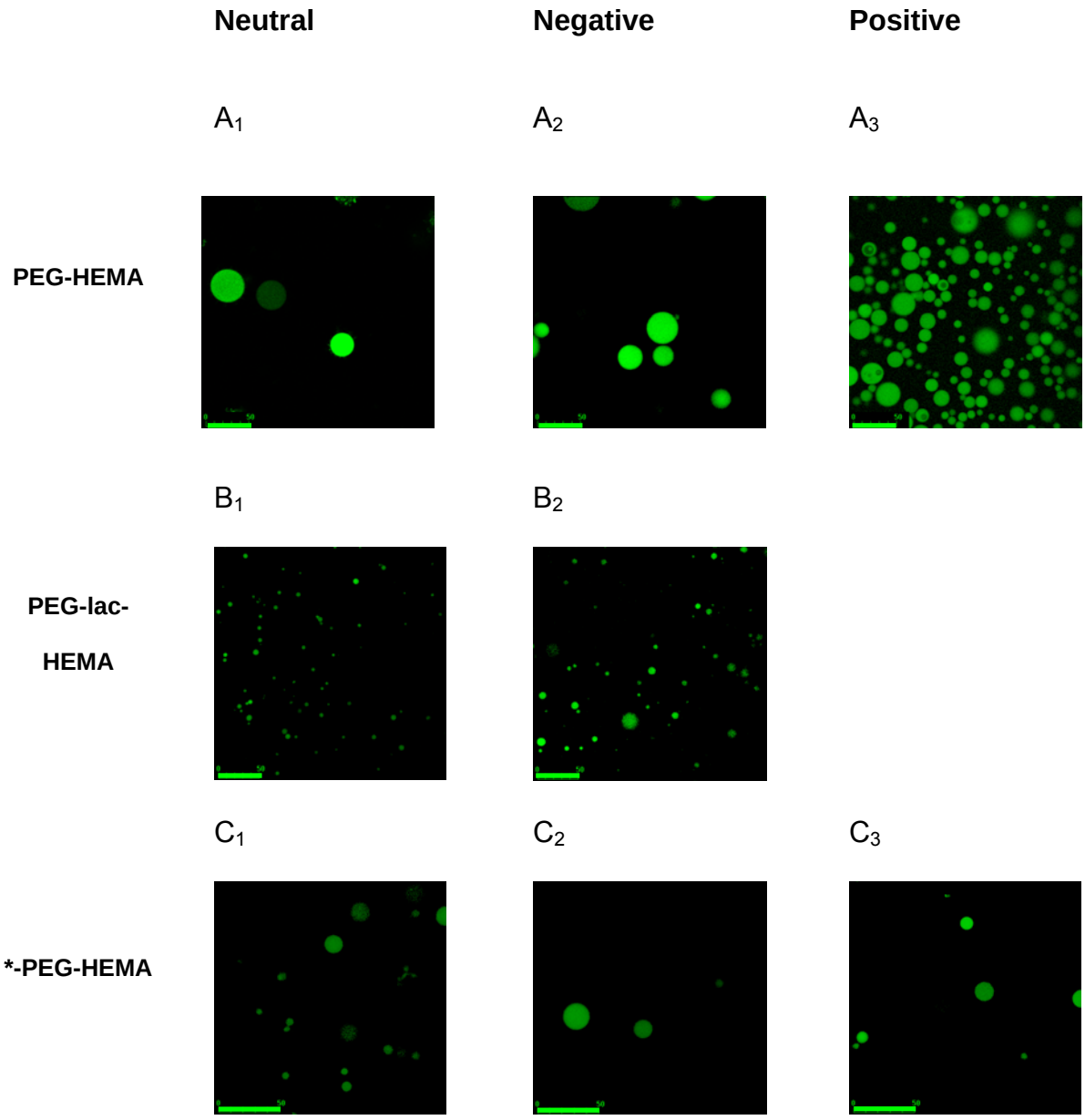

Figure 4. CLSM images of naked (A) PEG-HEMA, (B) PEG-lac-HEMA and (C) ✳-PEG-HEMA microgels. Subscript 1 refers to neutral gels, subscript 2 refers to negatively charged microgels (which contain MAA) and subscript 3 refers to positively charged microgels (which contain DMAEMA). The microgel core was fluorescently labeled with FITC-PEG. Scalebars represent 50 µm

Lipid coating of PEG based microgels. In a first approach we tried to coat the PEG microgels by a method previously reported by Kiser et al.[41,42]. Therefore (neutral) PEG microgels were sedimented on a (neutral) lipid film, however, lipid-coated PEG microgels could be hardly detected by CLSM. Subsequently, following the strategy of Moya et al.[43,44], positively and negatively charged PEG microgels were mixed with a suspension of oppositely charged liposomes. CLSM clearly

revealed that all the PEG microgels became lipid-coated in this way (Figure 5A, B and C).

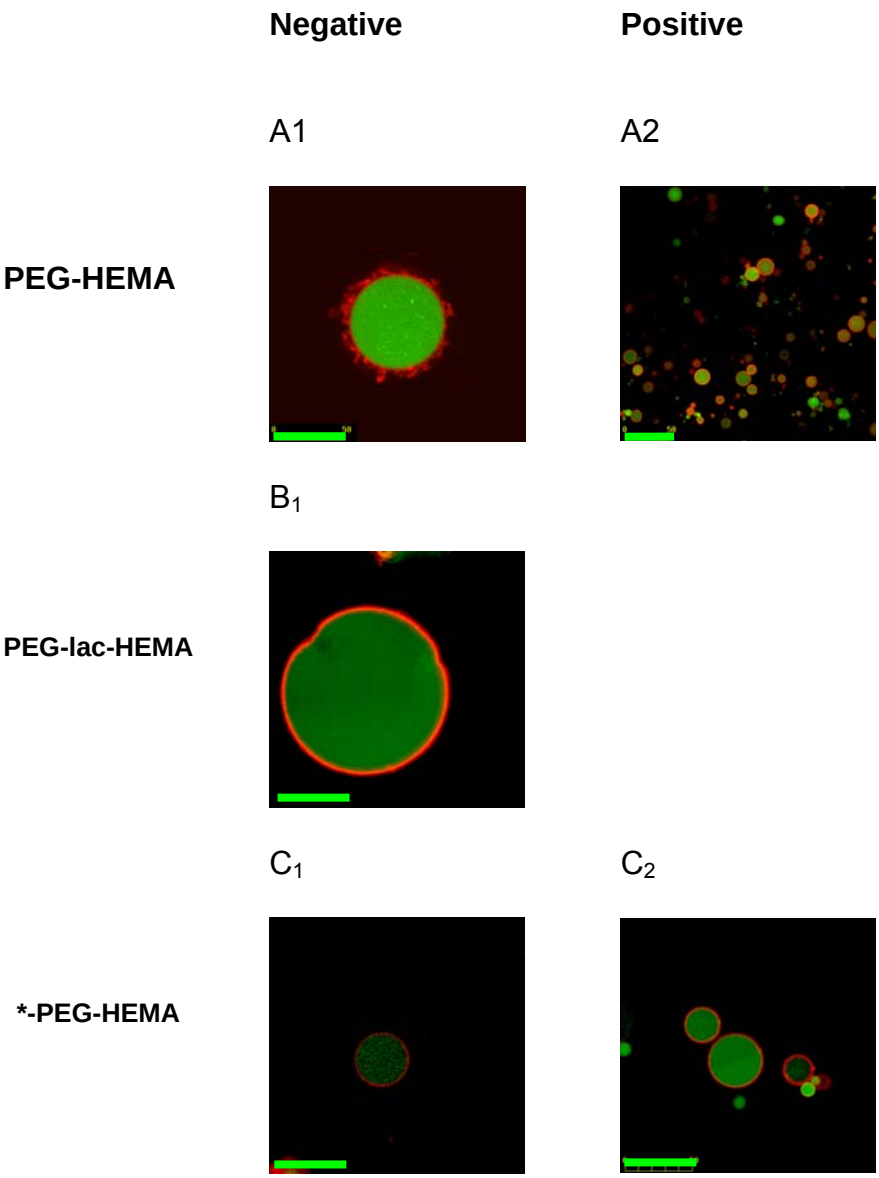

Figure 5. CLSM images of lipid-coated (A) PEG-HEMA, (B) PEG-lac-HEMA and (C) ✳-PEG-HEMA microgels. Subscript 1 refers negatively charged microgels (which contain MAA) and subscript 2 refers to positively charged microgels (which contain DMAEMA). Negative microgels were coated with DOPC:DOTAP (molar ratio 9:1) while positive microgels were coated with DPPC:DPPG:CHOL (molar ratio 4:1:5). The lipid membrane and microgel core were fluorescently labeled with respectively 5 mol% rho-DOPE and FITC-PEG. Scalebars represent 50 µm.

Release from PEG based microgels. Figure 6 shows the release of FITC-PEG from degrading PEG-HEMA, PEG-lac-HEMA and ✳-PEG-HEMA microgels. The release from both lipid-coated as well as naked (i.e. without lipid coating)

microgels was investigated. The inset tabulates the time it takes to release approximately 50% of the entrapped FITC-PEG ($t_{1/2}$).

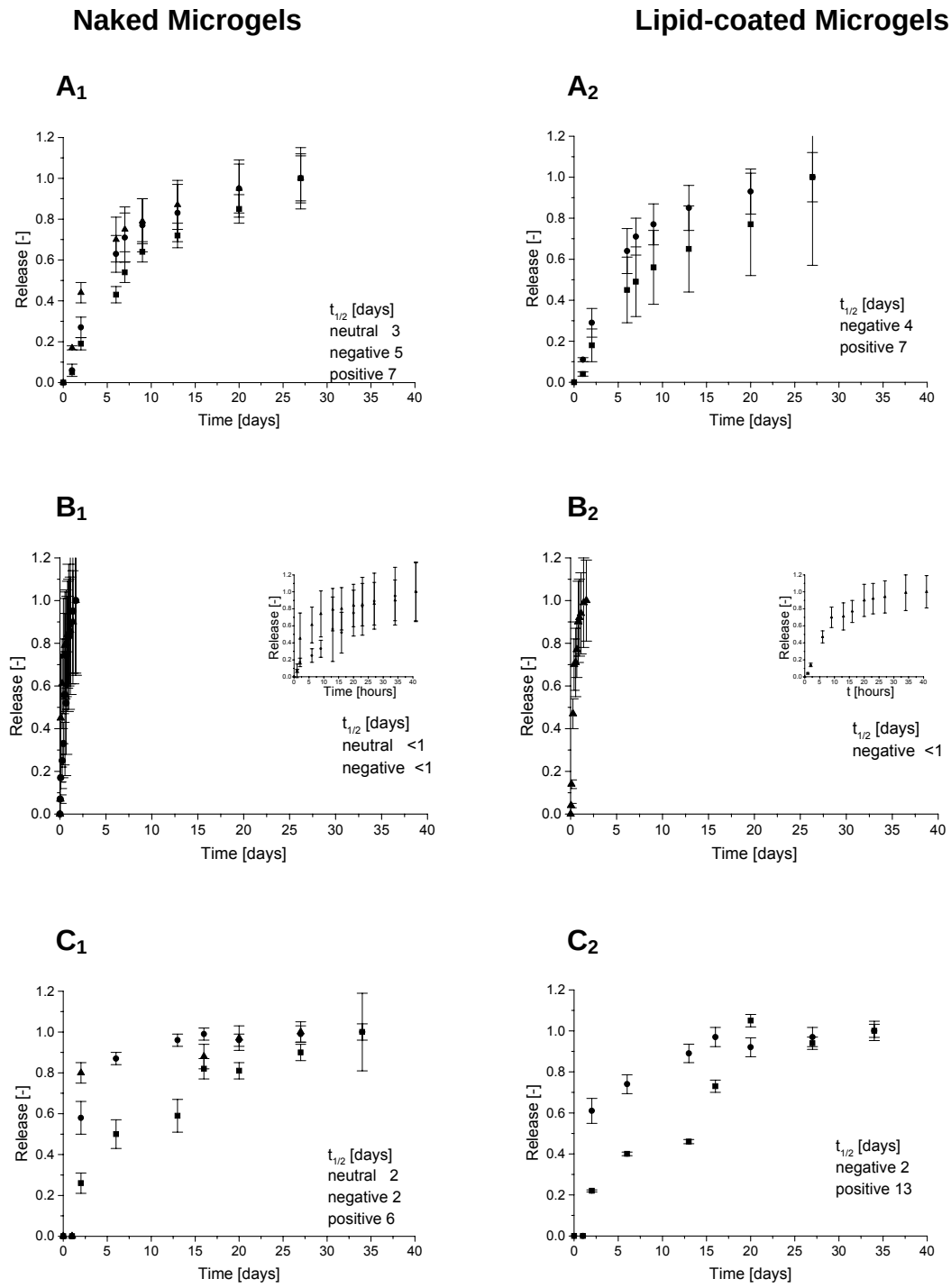

Figure 6. Release of FITC-PEG from degrading naked and lipid-coated PEG-HEMA (A), PEG-lac-HEMA (B) and ✳-PEG-HEMA (C, DS 3.8) microgels with (▲) neutral, (●) negative or (■) positive charge. Negative microgels were coated with DOPC:DOTAP (molar ratio 9:1) while the positive ones were coated with DPPC:DPPG:CHOL (molar ratio 4:1:5). 100% release corresponds to the amount of FITC-PEG released at the end of the experiment, when the particles were completely degraded.

PEG-HEMA microgels deliver the encapsulated FITC-PEG over a period of approximately one month (Figure $6A_1$). Generally speaking, as Figure $6A_1$ shows, the release of FITC-PEG from (neutral) PEG-HEMA, (negatively charged) PEG-HEMA-MAA and (positively charged) PEG-HEMA-DMAEMA microgels occurs similarly. Figure $6B_1$ shows that the release of FITC-PEG from neutral and charged PEG-lac-HEMA microgels is accomplished within hours, in agreement with the findings in Figure 3B which shows that PEG-lac-HEMA hydrogels degrade much faster than PEG-HEMA hydrogels. Figure $6C_1$ shows the results of FITC-PEG release from ✳-PEG-HEMA microgels. The release from the positively charged ✳-PEG-HEMA microgels seems to occur much slower than from the corresponding neutral and negative ✳-PEG-HEMA microgels.

Figure $6A_2$, B_2 and C_2 shows the release of FITC-PEG from degrading *lipid-coated* PEG-HEMA, PEG-lac-HEMA and ✳-PEG-HEMA microgels. The release of FITC-PEG from the lipid-coated PEG microgels seems similar to the release from the corresponding naked (i.e. non-coated) microgels (Figure $6A_1$, B_1 and C_1), indicating that the surrounding lipid membrane does not keep the FITC-PEG in the interior of the degrading PEG microgels.

PEG based nanogels

Synthesis of PEG-HEMA nanogels. As outlined in the introduction, as we are interested in degrading gel particles which can be taken up by cells for intracellular release of encapsulated drugs, we subsequently aimed to design PEG based nanogels, approximately one order of magnitude smaller than the PEG microgels described above. In chapter 2 we reported on the synthesis of biodegradable dextran nanogels, of about 400 nm in size[45]. We showed that lipid-coated dextran nanogels can be prepared by UV polymerization of dex-HEMA containing liposomes which were obtained by hydrating a lipid film with a dex-HEMA solution. “Naked” dextran nanogels (i.e. without lipid coating) were prepared by removing the lipid coating by TX 100. Using the liposome reactor technology, in this study we aimed to obtain PEG-HEMA nanogels.

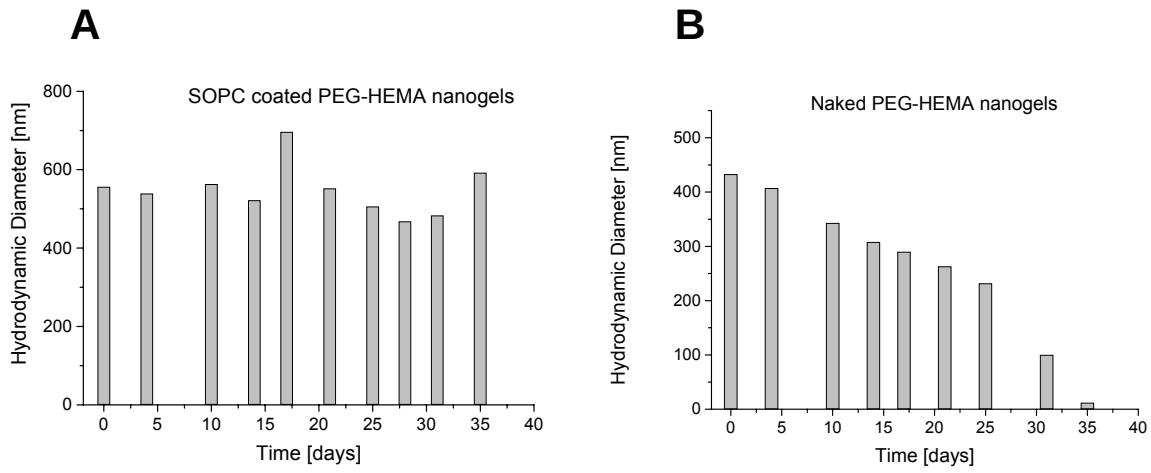

Figure 7. Hydrodynamic diameter, as measured by DLS, of (A) SOPC coated PEG-HEMA nanogels and (B) naked PEG-HEMA nanogels stored in PB (pH 8.5) at 37°C. To prepare the coated PEG-HEMA nanogels the SOPC lipid film was hydrated with a 20% (w/w) PEG-HEMA solution. To prepare the naked nanogels, the coated nanogels were exposed to TX 100. The shown data are the result of one set of experiments. Repeated experiments revealed the same results.

Figure 7A shows the outcome of DLS measurements on lipid-coated PEG-HEMA nanogels stored in buffer (pH 8.5) at 37°C. The PEG-HEMA nanogels were obtained by hydrating a SOPC lipid film with a PEG-HEMA solution. While the DLS measurements on the naked PEG-HEMA nanogels revealed that the naked nanogels get smaller in time (Figure 7B), clearly indicating degradation, we observed that the size of the lipid-coated nanogels (Figure 7A) did not decrease in time. One could wonder whether the PEG-HEMA gel in the lipid-coated particles did degrade. To examine this, we added TX 100 to the lipid-coated particles which were already in dispersion for 35 days. After removing the lipid coating, nanoparticles were no longer detected by DLS, proving that the nanogels in the interior of the liposomes were indeed degraded.

BSA release from degrading PEG-HEMA nanogels. We wondered whether (i) it is possible to load the PEG-HEMA nanogels with a model protein (BSA) and (ii) how the release of an encapsulated protein from PEG-HEMA nanogels looks like. Before measuring the release of BSA we characterized the BSA loaded PEG-HEMA nanogels by DLS.

Figure 7A shows the outcome of DLS measurements on liposomes obtained by hydrating a SOPC lipid film with a PEG-HEMA solution containing BSA. The PEG-HEMA filled liposomes are approximately 400 nm in size which is in accordance with the size of the pores of the used extrusion membrane, and show a rather narrow size distribution. Also, polymerization of the PEG-HEMA/BSA solution to obtain lipid-coated (BSA containing) PEG-HEMA nanogels did not alter the size and polydispersity of the particles (Figure 7B). To obtain naked PEG-HEMA/BSA

nanogels the lipid coating was removed by adding TX 100. Figure 7C shows the presence of about 400 nm sized naked PEG-HEMA/BSA nanogels, besides the presence of micelles (about 12 nm in size, as formed by TX 100 and SOPC lipids).

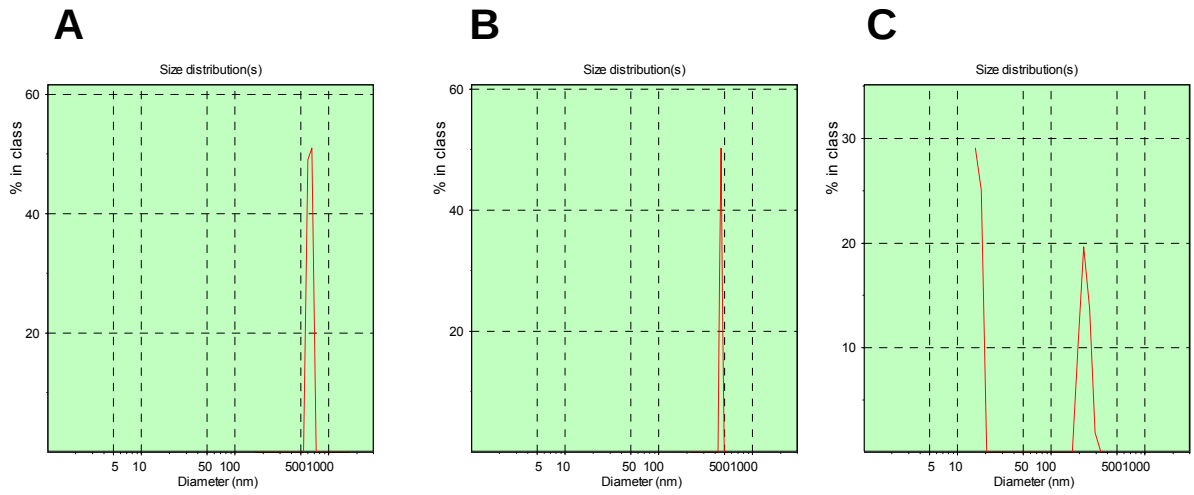

Figure 8. Size distribution, as measured by DLS, of SOPC coated PEG-HEMA nanogels loaded with BSA. A 20% (w/w) PEG-HEMA solution was used to hydrate the SOPC lipid film. (A) shows the SOPC liposomes before polymerization of the PEG-HEMA, (B) shows the SOPC coated PEG-HEMA nanogels after polymerization and (C) shows the particles after removal of the lipid coating.

Figure 9 shows the release of BSA from degrading PEG-HEMA nanogels. The following observations are made. First, the release of BSA starts immediately: after three days more than 50% of the BSA has been released. Second, after approximately 7 days BSA release does no longer occur, although dex-HEMA nanogels could still be detected by DLS (even up to 35 days, see Figure 7B). Release from degrading hydrogel matrices may occur diffusion-controlled and/or degradation-controlled. Highly likely, the release of BSA from the PEG-HEMA nanogels is controlled by the degradation of the PEG-HEMA matrix. Cruise at al estimated the pore size of PEG-diacrylate hydrogels (4kDa, 20%, in which BSA could not permeate) to be 2.2 nm[46]. Subsequently we assume that the average pore size of the intact (i.e. not yet degraded) PEG-HEMA matrix in the nanogels is smaller than the size of BSA molecules (i.e. 7.2 nm[5]). When degradation proceeds, the average pore size increases resulting in release of BSA. A third observation of Figure 9 is that the release of BSA from the lipid-coated and naked PEG-HEMA nanogels occurs similarly. This is rather surprising since we observed in chapter 3 that BSA is not released from SOPC liposomes filled with a BSA solution[8]. The following consideration may be important to explain our observations. First, degradation of a PEG-HEMA gel results in a PEG solution. As we showed in chapter 5, this raises the osmotic pressure[35]. Subsequently, the increase in the osmotic pressure of the gel core in the lipid-coated PEG-HEMA nanogels may deform (and/or destroy) the lipid coating which allows the release of BSA into the medium.

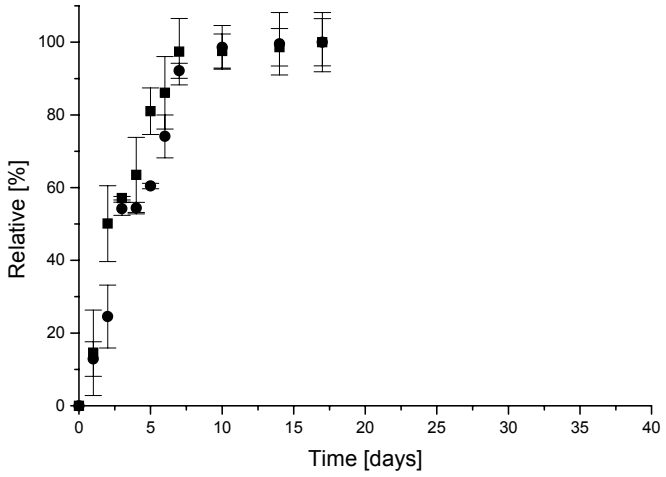

Figure 9. Cumulative release of BSA from degrading PEG-HEMA nanogels with (■) or without (●) SOPC coating. The lipid coating was hydrated with a 20% (w/w) PEG-HEMA solution. 100% release corresponds to the amount of BSA released when the nanogels were completely degraded (i.e. when nanogels could no longer be detected by DLS).

CONCLUSIONS

In this study PEG-HEMA, PEG-lac-HEMA and ✳-PEG-HEMA hydrogels were investigated. Rheological measurements on hydrogel slabs showed that PEG-lac-HEMA matrices degrade very fast (in terms of days), while it takes about one month for linear PEG-HEMA and several months for ✳-PEG-HEMA hydrogels to become degraded. We showed that, under all aqueous conditions PEG-HEMA, PEG-lac-HEMA and ✳-PEG-HEMA microgels can be obtained by emulsion polymerization, making use of the principle of the immiscibility between dextran and PEG. Positively and negatively charged PEG microgels could be lipid-coated by mixing them with oppositely charged liposomes. While the applied lipid coating did not influence the release of fluorescently labeled PEG from the microgels, the release was clearly influenced by the type of methacrylated PEG used (lasting from hours to months). Lipid-coated PEG-HEMA nanogels were obtained by UV polymerization of a PEG-HEMA solution in the interior of liposomes. While it took about 1 month for complete degradation of the PEG-HEMA nanogels, as measured by dynamic light scattering, encapsulated BSA was completely released after 7 days.

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BACKGROUND AND SUMMARY

Background

The last years the development of macromolecular drugs in the combat against hereditary sicknesses or cancer has gained much of attention. Potential of these peptides, proteins or (oligo)nucleotides would be infinite, if it wasn't for some major problems that showed up along the way.

A first problem is the stability of these molecules once entered the body. Because of the presence of several enzymes and low pH (for example in the stomach), these therapeutic molecules are degraded and loose their therapeutic activity. A second problem is that most of these medicines are aggressive and damage not only the target cells, but also the surrounding healthy cells causing severe side effects. Last, the particles are removed too quick from the body: small particles are removed mostly by renal clearance, larger particles are taken up by phagocytosis. As a results permanent administration or frequently repeated injections are necessary, causing patients incompliance.

To cope with all these problems, several different carrier systems are under development last decade. These drug delivery systems can bring the therapeutic molecules to a specific target during a sustained time interval. Hydrogels are very suited for this purpose: they form a network from mutually cross-linked polymers and contain high amounts of water. Especially biodegradable hydrogels are suited as a drug delivery system. In the first place because no (surgical) intervention is needed to remove the system and in the second place because it is possible to govern the drug release from it by altering the cross-link density of the hydrogels.

Many of the described molecules have intracellular targets, often in the nucleus. To get there, the particles must meet some conditions. The most important criterium is that they must be small enough. On the one hand the particles can be administered parenterally, they may not aggregate as this can block blood flow and they must be able to penetrate in cells. Nanoscopic particles are most suited for this purpose. On the other hand, particles can be administered locally, releasing the drugs over a sustained time interval without entering the bloodstream. In this case, microscopic particles are most suited.

Besides "being small enough" the particles must also fulfil some other criteria in order to be applicable: (i) they must encapsulate the therapeutic molecules in an efficient way without causing any damage during the production process, (ii) in the body, the encapsulated molecules must be protected against degradation and the particles may not aggregate, (iii) the therapeutic molecules must be released in an appropriate way, without burst release and of course (iv) the composing materials must be non-toxic and preferably biodegradable in the human body.

As mentioned earlier, many of these therapeutics are too toxic for “healthy” cells. A possibility to save these cells is to encapsulate the active molecules in drug carrier systems and to target them directly to the target cells. As a result, damage of healthy cells is avoided and less drugs have to be administered. To types of targeting are known: active and passive targeting.

As mentioned above, particles are removed from the bloodstream quickly by phagocytosis and the drugs will end up in the organs of the reticulo endothelial system (RES) like the liver, spleen and lymph. When these organs were not the organs of interest, the drugs are lost and in addition liver, spleen and lymph can be severely damaged. As a result, it would be appropriate to avoid phagocytic uptake. Phagocytes recognise foreign particles after binding of opsonines at the particles’ surface. One of the most commonly used methods to avoid the binding of opsonines at the particles’ surface –and as a result camouflage the particles against phagocytosis- is by covering the particles’ surface with poly(ethylene glycol). The particles can escape from the phagocytes and circulate longer in the bloodstream. Finally, they will escape from the bloodstream through leaky vasculature, which is typically for tumours, inflammation tissue or infarcted areas. As a consequence, PEGylated particles will end up in this type of tissue. This process is called passive targeting. PEGylation has an additional advantage: the body hardly recognizes these foreign objects and almost no allergic or rejection reactions occur.

A second way of targeting is the so-called active targeting. The surface of the particles is provided with ligands that can only bind with receptors that reside on the surface of specific target cells.

Particles that are large enough are taken up by endocytosis. De content of the endosomes is degraded in the lysosomes. It is well known however that PEGylated particles hardly escape from these endosomes and as a result they end up in lysosomes were they are degraded by enzymes before they have delivered their therapeutic content in the cytosol. Hence it would be ideal if the nanogels could lose their PEGylated lipid coating after they are incorporated in the endosomes. Fortunately the endosomes show an intrinsic property which can help: the pH in the endosomes is much lower than in the rest of cell.

In this thesis it was our intention to develop nanoscopic- and microscopic drug delivery systems composed of hydroxyethyl methacrylated dextran and poly (ethylene glycol) (respectively dex-HEMA and PEG-HEMA) in order to develop a sound formulation for long-term drug delivery. Dex-HEMA and PEG-HEMA solutions form hydrogels upon radical polymerization: hydrolysable cross-links are formed. Hydrolysis of the formed hydrogel occurs under physiological conditions at the carbonate esters resulting in dextran or PEG and some poly(HEMA) fragments. The higher the amount of cross-links, the higher the cross-link density and the longer it takes to come to complete degradation of the hydrogels. The cross-link density can be adapted simply by varying the number of HEMA groups on the dextran- or PEG

backbone. This way hydrogels can be prepared, entrapping drug molecules in their polymer lattice, releasing the drugs rapidly or slowly- depending on the cross-link density.

Moreover we wanted provide these nano- and microgels with a lipid coating. This can be a convenient tool to adapt the hydrogel surface. First PEGylated lipids can be inserted to make particles which are possible to escape from phagocytes. Secondly, pH sensitive lipids can be inserted. This way the (PEGylated) lipid coated particles can loose their coat after they arrive in the acidic environment of the endosomes and as a consequence it is easier to escape to the cytosol before they are degraded in the lysosome. A last possibility, which was not carried out in this work, is inserting lipids conjugated with specific ligands that can only bind to specific target cells.

In this work some physicochemical properties of PEG were studied too, since it proposes of some properties that are -to our opinion- ideal to make it as a nanoscopic or microscopic drug delivery system. Some new polymerizable PEG molecules were prepare with this aim.

Summary

In the **first chapter** an overview is given of the published literature concerning sustained drug delivery from nanosized matter. Both liposomes and nanoscopic hydrogel systems were discussed. Liposomen are hollow lipid vesicles filled with water. This unique structure offers the possibility to accommodate both hydrophilic and hydrophobic molecules, respectively in the aqueous core and in the lipid shell (see chapter 1 Figure 1). Drug molecules escape generally from the liposomes by diffusion. At present liposomal formulations for the sustained delivery of some chemotherapeutics are already FDA approved and several others are in the pipe line to commercial use.

Nanogels on the other hand are no hollow structures, but are formed by a network of polymers filled with water in which drugs can be included. (see chapter 1 Figure 3). Consequently they can hold more molecules and the release can be controlled more easily. A wide range of different materials has been used to produce nanogels, ranging from synthetic polymers, among which poly(lactate-co-glycolide) is by far used, to some naturally occurring sugars like for example chitosan.

Moreover, it is possible to provide nanogels with a coating which can do service as extra barrier against burst release or can help with active or passive targeting. Some examples of coated nanogels were also discussed in the first chapter.

In the **second chapter** we describe how nanoscopic particles can be produced which are mainly composed of dextran. These nanogels are produced making use of liposomes as a nanoscopic mould: the liposomen are filled by hydrating a lipid film with a polymerizable dex-HEMA solution. Afterwards dex-HEMA

is cross-linked by means of UV. The result is a dextran nanogel surrounded by a double lipid layer, which was shown by dynamic light scattering, atomic force microscopy and electron microscopy. Moreover the lipid layer can be removed from the nanogels by adding a detergent. As discussed above, dex-HEMA hydrogels contain hydrolysable carbonate esters. It was shown by means of dynamic light scattering that these nanogels degrade also under physiological circumstances and – by increasing the cross-link density- the particles degraded during 5 up to 18 days. As our aim was to make a drug delivery systems which delivers its content intracellularly, it was also evaluated whether these particles can be taken up by cells. By means of confocale microscopy we could confirm that coated nanogels are indeed taken up VERO cells.

In the **third chapter** it is described how model proteins, bovine serum albumin (BSA) and lysozyme, can be included in both the coated and naked nanogels. This was done by administering them to the polymer solution that was used to hydrate the lipid film. Afterwards the solution was polymerized in the cavity of the liposomes and as a result the proteins were included in the dex-HEMA nanogels. When degradation occurs, the cross-links detach and the pores of the network enlarge. When the pores become larger than the dimensions of the included proteins, these proteins are released in the surrounding medium. The release rate of the included molecules depends on the size of the molecule, on the cross-link density of the nanogel and on the presence of a lipid coating around the nanogels, provided that the incorporated molecules are sufficiently large. A disadvantage of this system is that the proteins are already present during the polymerization process. However we found out that lysozyme could preserve up to 75% of its original activity. With a view to long-term storage we freeze-dried the nanogels and showed that this has no influence on the degradation profile of the particles. It is also of vital importance that the nanogels do not aggregate in the body, as this can cause obstruction in the blood capillaries. To examine this the behaviour of coated nanogels in human serum was examined. When the nanogels are sufficiently diluted, the particles did not aggregate.

As already mentioned above, drug delivery systems for parenteral administration are often provided with a PEG coating, this to avoid rapid removal from the blood by phagocytosis. In the **fourth chapter** PEGylated dex-HEMA nanogels were prepared by use of liposomes in which PEGylated lipids were incorporated (chapter 4 Figure 2). The surface charge of these particles is lower than particles without a surrounding (neutral) PEG layer, which can be explained by the screening effect of the PEG molecules. This reduced surface charge, however, caused a somewhat lower binding to the cell membrane, the first step in the process of endocytosis. Fortunately, it was also shown that this reduced cell binding has hardly any influence on the cellular uptake of the particles: PEGylated nanogels are taken up as efficient as non-PEGylated particles by VERO and RPE cells. Besides, the PEGylated particles do not show aggregation in human serum. It was already

mentioned that PEGylated particles hardly escape from the endosomes, and as a result they often end up in the lysosomes were they are enzymatically degraded. For this reason nanogels were developed with pH sensitive PEGylated lipid coating. The pH sensitive lipid is a synthetic diplasmenylcholine that is hydrolysed at pH 4-5 producing free fatty acids. These fatty acids ensure that the lipid coating is destabilised and detaches from the nanogels (see chapter 4 Figure 4), which was shown visually as well as by dynamic light scattering.

In the **fifth chapter** PEG was examined as a possible polymer to produce drug delivery systems. The reason to examine PEG is that our research group developed earlier 'exploding dex-HEMA microgels' (see chapter 5 Figure 1). These microgels exist of a dex-HEMA core surrounded by a lipid or polymeric shell. During degradation of dex-HEMA the osmotic pressure is build up. Initially this pressure remains fairly low, but at the end it shows a sudden increase. When microgels composed of dex-HEMA and surrounded by a lipid or polymeric shell are produced, this remarkable pressure profile of dex-HEMA causes a sudden rupture of the surrounding coating and as a result the included drug molecules are released suddenly. To avoid that the included molecules are released too rapidly (due to leaking for example), it is necessary to use strong membranes, but the pressure needed to rupture these membranes is often not reached by dex-HEMA. It is well known that the osmotic pressure of PEG is higher than the osmotic pressure of dextran. Hence ours interest in PEG: when higher osmotic pressure is reached, also stronger membranes can be disrupted. In analogy with dex-HEMA also PEG was provided with a polymerizable group, resulting in hydroxyethyl methacrylated PEG (PEG-HEMA).

To examine the pressure profile of PEG-HEMA, an earlier described method was used. In this method, non degraded PEG-HEMA hydrogels containing free PEG (degradation product) were used to mimic degrading PEG-HEMA hydrogels. This way it was shown that degrading PEG-HEMA hydrogels show similar osmotic behaviour as dex-HEMA, but much higher pressure peaks were recorded.

To obtain a better insight in the contribution of free chains to the osmotic pressure we have examined the impact of the molecular weight of the free chains in dex-HEMA and PEG-HEMA hydrogels. We revealed that the difference between the contributions of network chains and free chains to the osmotic pressure decreases with increasing molecular weight. Higher molecular weight free chains behave as network chains.

In **chapter six** both microscopic and nanoscopic hydrogel particles were made composed of PEG. Two new polymerizable PEG polymers were produced: on the one hand PEG-lactate-HEMA, which degrades more rapidly than PEG-HEMA thanks to its lactate spacer and on the other side star-shaped PEG-HEMA (✳-PEG-HEMA). This eight armed molecule was provided with polymerizable hydroxyethyl

methacrylate. This way ✳-PEG-HEMA contains more polymerizable groups then PEG-HEMA and the corresponding hydrogels degrade significantly slower.

By means of emulsion polymerization it is possible to produce PEG-HEMA, PEG-lac-HEMA and ✳-PEG-HEMA microgels. When they are co-polymerized with a positive or negative charged methacrylate we can provide them with a lipid coating. The coating process is based on electrostatic interactions between the charged microgels and the opposed charged lipid membrane. The lipid coated microgels could be visualised by means of confocal microscopy. To examine the ability of these (lipid coated) PEG microgels as a drug delivery system, the release profile of FITC-PEG was evaluated. Encapsulated molecules are released fastest when the microgels were made of PEG-lac-HEMA: already after some hours the release has been completed. ✳-PEG-HEMA microgels on the other hand deliver their content slowest: the release of FITC-PEG lasts for a month. PEG-HEMA microgels release their content during a almost three weeks. Although the lipid coating has hardly any influence on the release profile, it might be convenient to provide the microgels with a coating as described in chapter 4.

In analogy with dex-HEMA nanogels described in chapter 2-4, PEG-HEMA nanogels are also made based on the same method: a lipid film was hydrated with a PEG-HEMA a solution and afterwards polymerized by use of UV. When compared to dex-HEMA nanogels, the degradation of PEG-HEMA nanogels is much slower, which was also seen on macroscopic scale in chapter 5: complete degradation of the nanogels takes 35 days. Encapsulated BSA is more rapidly released than expected: after 7 days all included BSA has been released.

ACHTERGROND EN SAMENVATTING

Achtergrond

De laatste jaren is de ontwikkeling van macromoleculaire geneesmiddelen in de strijd tegen erfelijke ziekten, kanker of hart- en vaatziekten enorm toegenomen. Het potentieel van deze peptiden, eiwitten of (oligo)nucleotiden zou haast oneindig zijn, ware het niet dat er zich nog verschillende problemen voordoen.

Een eerste probleem schuilt in de stabiliteit van het geneesmiddel wanneer het toegediend wordt in het lichaam. Door de aanwezigheid van enzymen of door een verlaagde pH (bijvoorbeeld in de maag) worden deze therapeutische moleculen afgebroken en als gevolg onbruikbaar. Een tweede probleem is dat bepaalde geneesmiddelen agressief zijn en niet alleen het doelweefsel beschadigen, maar ook alle andere cellen die ze op hun weg tegenkomen en daardoor voor ernstige neveneffecten kunnen zorgen. Ten slotte worden deze geneesmiddelen zeer snel uit het lichaam verwijderd: wanneer ze voldoende klein zijn gebeurt dit voornamelijk door renale klaring, grotere moleculen worden vaak opgenomen door fagocyterende cellen. Bijgevolg zijn continue toediening of toediening via herhaalde injecties noodzakelijk, en dit tot ongemak van de patiënt.

Om al deze problemen het hoofd te kunnen bieden, zijn er de laatste decennia verschillende dragersystemen voor macromoleculaire farmaca ontwikkeld die in staat zijn het geneesmiddel doelgericht af te geven aan een bepaald celtype en dit bovendien doen over een bepaalde tijdspanne. Voor de vervaardiging van deze dragersystemen zijn hydrogels zeer geschikt. Hydrogels bestaan uit een netwerk gevormd door onderling verbonden polymeren die een grote hoeveelheid water vasthouden. Vooral biodegradeerbare hydrogels zijn geschikt als afgiftesysteem. Ten eerste omdat ze, dankzij hun degradeerbaarheid, niet (chirurgisch) verwijderd moeten worden, en ten tweede omdat het mogelijk is om de vrijstelling van de ingesloten geneesmiddelen te sturen, onder andere via het aanpassen van de vernettingsdichtheid.

Veel van de beschreven macromoleculaire geneesmiddelen moeten in de cel terechtkomen, heel vaak zelfs in de celkern. Om hier te geraken moeten de afgiftesystemen aan verschillende voorwaarden voldoen. De voornaamste voorwaarde is dat ze klein genoeg moeten zijn. Enerzijds kunnen de partikels parenteraal toegediend worden en dan moeten ze in de bloedbaan kunnen circuleren zonder verstoppingen te veroorzaken en kunnen binnendringen in de cellen. Hiervoor zijn nanoscopisch kleine deeltjes het meest geschikt. Anderzijds kunnen de partikels lokaal worden toegediend, daar blijven zonder in de bloedbaan terecht te komen en het geneesmiddel gedurende lange tijd vrijgeven. Microscopisch kleine deeltjes zijn hiervoor meer geschikt.

Naast “voldoende klein zijn” moeten deze afgiftesystemen nog aan enkele andere criteria voldoen om toepasbaar te zijn: (i) ze moeten de geneesmiddelen

efficiënt insluiten zonder ze te beschadigen tijdens het productieproces, (ii) in het lichaam moeten de ingesloten geneesmiddelen beschermd worden tegen afbraak en de partikels mogen niet aggregeren, (iii) de geneesmiddelen moeten op een geschikte manier vrijgesteld worden, zonder initieel een verhoogde vrijstelling te vertonen en uiteraard (iv) mag het materiaal waaruit de drager gemaakt is niet toxisch zijn en bij voorkeur afbreekbaar in het lichaam.

Zoals reeds eerder gesteld zijn vele geneesmiddelen te toxisch voor “gezonde” cellen en het is bijgevolg van groot belang om deze zoveel mogelijk te sparen. Een mogelijkheid is om de actieve stof te encapsuleren en ze specifiek naar de doelcellen te richten. Op deze manier worden niet alleen nefaste gevolgen vermeden voor gezonde cellen, maar moet ook minder geneesmiddel worden toegediend. Dit zogenaamde targetten kan op verschillende manieren gebeuren: passief of actief.

Zoals reeds beschreven zullen partikels die zich in de bloedbaan bevinden snel uit het lichaam verwijderd worden door fagocytose en zullen de ingesloten geneesmiddelen bijgevolg in de organen van het reticulo endotheliaal stelsels (RES) zoals de lever, de milt of het lymfesysteem terechtkomen. Wanneer dit niet de doelcellen waren, is het geneesmiddel onherroepelijk verloren en kunnen deze organen bovendien schade oplopen. Het zou dus ideaal zijn, mocht het mogelijk zijn om het proces van fagocytose te vermijden. Fagocyterende cellen herkennen lichaamsvreemde objecten doordat zogenaamde opsonines binden aan het oppervlak van deze partikels. Eén van de meeste gebruikte manier om het binden van deze opsonines te verhinderen -en aldus de partikels te camoufleren voor fagocyten- is het oppervlak van de partikels bedekken met poly(ethyleen glycol) (PEG). Zodoende krijgen de partikels de kans om te ontsnappen aan fagocyten en kunnen ze langer in de bloedbaan circuleren. Daarna kunnen ze eruit ontsnappen via *fenestrae* (“gaatjes”) in de vasculatuur, hetgeen typisch voorkomt bij tumoren of beschadigd weefsel. Bijgevolg zullen gePEGyleerde partikels uiteindelijk terechtkomen in het doelweefsel. Dit noemt men *passief targetten.* PEGyleren van partikels heeft een bijkomend voordeel: het lichaam herkent deze vreemde objecten nagenoeg niet en bijgevolg treden er weinig allergische reacties of afstotingsverschijnselen op.

Een tweede manier om enkel doelcellen te bereiken is via het zogenaamde *actief targetten*. Het oppervlak van de partikels wordt voorzien met bepaalde liganden die binden met receptormoleculen die specifiek voorkomen op het oppervlak van doelcellen.

Partikels die voldoende groot zijn, worden door de cel opgenomen via endosomen. De inhoud van de endosomen wordt afgebroken in de lysozomen. Nu is het al een tijdje bekend dat gePEGyleerde partikels moeilijk ontsnappen uit endosomen, waardoor ze vaak eindigen in lysozomen en bijgevolg de bio-actieve moleculen afgebroken zijn nog voor hun vrijgave in de cel. Vandaar dat het ideaal

zou zijn, mochten de nanogels hun gePEGyleerde lipid coating verliezen nadat ze opgenomen zijn in de endosomen. Gelukkig vertonen de endosomen een intrinsieke eigenschappen die hierbij kan helpen: de pH in het endosoom is veel lager dan in de rest van de cel.

In deze thesis was het de bedoeling om nanoscopische en microscopische dragersystemen van hydroxyethyl gemethacryleerd dextraan en poly(ethyleen glycol), respectievelijk dex-HEMA en PEG-HEMA genoemd, te bestuderen met het oog op de ontwikkeling van een degelijke formulatie voor vertraagde geneesmiddelen vrijgave. Dex-HEMA en PEG-HEMA oplossingen kunnen gepolymerizeerd worden onder invloed van radicalen. Ze vormen hydrogels die afbreken onder fysiologische omstandigheden: ter hoogte van de carbonaatesters komen de knooppunten los en wordt dextraan en kleine fragmenten poly(HEMA) vrijgesteld. Hoe sterker de hydrogels vernet zijn, hoe te langer het duurt om de gels volledig af te breken. De vernettingsgraad kan eenvoudig worden aangepast door het aantal HEMA groepen op het polymeer te laten variëren. Op die manier kunnen hydrogels ontwikkeld worden waarin geneesmiddelen ingesloten zitten die – afhankelijk van de vernettingsgraad- traag of snel worden afgegeven.

We wilden bovendien deze nano- en microgels van een lipid coating voorzien. Dit kan een handig hulpmiddel zijn om het oppervlak van de gels naar wens aan te passen. Ten eerste kunnen er gePEGyleerde lipiden ingevoegd worden om gePEGyleerde partikels te maken, die kunnen ontsnappen een de fagocyterende cellen. Ten tweede kunnen pH gevoelige lipiden ingevoegd worden. Op deze manier kan de (gePEGyleerde) lipid coating loskomen van de partikels wanneer ze in het endosoom terecht komen en kunnen de deeltjes hierdoor gemakkelijker onsnappen zonder afgebroken te worden in het lysozoom. Een laatste mogelijkheid, die hier niet uitgevoerd werd, is het invoegen van lipiden waarop liganden gebonden zijn die binden aan specifieke receptoren die enkel op de doelcellen voorkomen.

In dit werk werden ook enkele fysicochemische eigenschappen van PEG bestudeerd, aangezien het naar onze mening enkele eigenschappen vertoont die het tot een ideaal materiaal maken voor gebruik als afgiftesysteem. Met dit doel werden enkele nieuwe polymerizeerbare PEG moleculen aangemaakt.

Samenvatting

In het **eerste hoofdstuk** wordt een overzicht gegeven van de reeds verschenen literatuur over vertraagde vrijstelling van geneesmiddelen uit nanoscopische systemen. Zowel liposomen als nanoscopische hydrogel systemen werden besproken. Liposomen zijn holle vesikels gevuld met water met een wand die bestaat uit een dubbele lipidenlaag. Deze unieke structuur biedt de mogelijkheid om zowel hydrofiele als hydrofobe moleculen te herbergen, respectievelijk in de waterige kern en in de wand (zie *hoofdstuk 1 Figuur 1*). De geneesmiddelen ontsnappen doorgaans uit de liposomen door diffusie. Momenteel zijn er reeds

liposoom formulaties in gebruik voor de toediening van chemotherapeutica en verschillende andere geneesmiddelen zitten in de pijplijn naar commercieel gebruik.

Nanogels daarentegen zijn geen holle structuren, maar bestaan uit een netwerk van polymeren gevuld met water waarin geneesmiddelen kunnen worden ingesloten. Bijgevolg kunnen ze veel méér moleculen vasthouden en de vrijgave ervan kan gemakkelijker gestuurd worden. Er is een waaier van verschillende materialen gebruikt om nanogels te produceren, gaande van synthetische polymeren, waaronder poly(lactaat-co-glycolide) veruit het meest gebruikt wordt, tot natuurlijk voorkomende suikers zoals chitosan.

Nanogels kunnen bovendien voorzien worden van een coating die dienst kan doen als extra barrière tegen vervroegde vrijstelling of die kan helpen bij actieve of passieve targetting. Ook hiervan werden enkele voorbeelden besproken in het eerste hoofdstuk.

In het **tweede hoofdstuk** wordt beschreven hoe nanoscopische partikels kunnen aangemaakt worden die voornamelijk bestaan uit dextraan. Deze nanogels worden gemaakt gebruik makende van liposomen als gietvorm: de liposomen worden gevuld door een lipide film te hydrateren met een polymerizeerbare dex-HEMA oplossing. Nadien worden ze vernet met behulp van UV bestraling. Als resultaat worden dextraan nanogels bekomen, omgeven door een dubbele lipid laag, hetgeen werd aangetoond door dynamische lichtverstrooiing, atomic force microscopy en elektronen microscopie. De lipid laag kan bovendien van de nanogels verwijderd worden door het toevoegen van een detergent. Zoals hierboven besproken bevatten dex-HEMA hydrogels hydrolyse gevoelige carbonaat esters. Via dynamische lichtverstrooiing werd nagegaan of deze nanogels ook afbreken onder fysiologische omstandigheden. Dit was inderdaad het geval en door verhoging van de vernettingsgraad kon de degradatieperiode waarover de partikels afbraken verlengd worden van 5 tot 18 dagen. Aangezien het de bedoeling is om afgiftesystemen te maken die het geneesmiddel uiteindelijk in de cel afgeven, werd ook geëvalueerd of deze partikels werden opgenomen door cellen. Via confocale microscopie werd bevestigd dat gecoate nanogels inderdaad worden opgenomen door VERO cellen.

In het **derde hoofdstuk** wordt beschreven hoe modeleiwitten, BSA en lysozyme, kunnen worden ingesloten in zowel de gecoate als in de niet gecoate nanogels. Dit lukt door ze toe te dienen aan de polymeeroplossing waarmee de lipid film gehydrateerd wordt. Daarna wordt de oplossing gepolymerizeerd binnenin het liposoom en zitten de eiwitten ingesloten in het dex-HEMA netwerk. Wanneer degradatie optreedt van dit netwerk en de mazen groter worden dan de afmetingen van de ingesloten eiwitten, komen deze eiwitten vrij. De vrijstelling van de ingesloten moleculen is afhankelijk van de grootte van het molecule, van de vernettingsgraad van de nanogel en van de aanwezigheid van een lipid coating rond de nanogels, op voorwaarde dat de ingesloten moleculen voldoende groot zijn. Een nadeel van deze

manier van insluiten is dat de eiwitten reeds aanwezig zijn tijdens het polymerizatieproces. Nochtans konden we aantonen dat lysozyme tot 75% van zijn originele activiteit kan behouden. Met het oog op langdurige opslag konden we ook aantonen dat vriesdrogen een optie is. De degradatie-eigenschappen van de nanogels veranderen hierdoor niet. Het is ook van cruciaal belang dat de nanogels niet gaan aggregeren eens ze in het lichaam zitten, dit zou immers verstopping van bloedvaten kunnen veroorzaken. Om dit na te gaan werd het gedrag van gecoate nanogels in humaan serum nagegaan. Wanneer de nanogels voldoende verdund zijn, treedt geen aggregatie op.

Zoals hierboven beschreven worden parenteraal toegediende dragersystemen vaak voorzien van een PEG coating, dit om te vermijden dat ze te snel uit de bloedbaan verwijderd zouden worden. In het **vierde hoofdstuk** worden dex-HEMA nanogels aangemaakt die voorzien zijn van een PEG coating. Dit gebeurt door in de gebruikte liposomen lipiden in te sluiten waaraan een PEG staart gekoppeld is. De oppervlaktelading van deze partikels is lager dan van partikels waar geen PEG coating rond zit, wat verklaard wordt door het afschermend effect van de neutrale PEG laag. Deze verminderde oppervlakte lading zorgt er wel voor dat de partikels minder goed binden aan het oppervlak van cellen, de eerste stap in het proces van endocytose. Gelukkig kon ook worden aangetoond dat deze verminderde binding nauwelijks invloed heeft op de opname van de partikels: gePEGyleerde nanogels worden net zo efficiënt opgenomen door VERO en RPE cellen als niet gePEGyleerde nanogels. Ze vertonen ook geen aggregatie in serum. Er werd eerder al vermeld dat gePEGyleerde partikels moeilijk ontsnappen uit het endosoom, waardoor ze uiteindelijk in de lysozomen belanden en afgebroken worden. Daarom werden nanogels ontwikkeld met een pH gevoelige gePEGyleerde lipid coating. Bij zure pH (zoals in het endosoom) komt de lipid coating los en zijn de partikels bijgevolg ge-dePEGyleerd. Hiertoe werden synthetisch aangemaakte diplasmenylcholines ingesloten. Deze pH gevoelige lipiden breken af bij pH 4-5 en stellen vetzuren vrij. Deze vetzuren zorgen ervoor dat de lipid coating gedestabiliseerd wordt en loskomt, wat zowel door dynamische lichtverstrooiing als visueel kon worden aangetoond.

In het **vijfde hoofdstuk** werd PEG onderzocht als mogelijk polymeer om geneesmiddelenafgiftesystemen te maken. De reden dat PEG onderzocht wordt is omdat door onze onderzoeksgroep reeds eerder exploderende dex-HEMA microgel partikels werden onderzocht. Deze micropartikels bestaan uit een dex-HEMA kern en zijn omgeven door een lipid of polymeer membraan (zie *hoofdstuk 6 Figuur 1*). Tijdens degradatie van dex-HEMA wordt een osmotische druk opgebouwd. Initieel blijft deze druk tamelijk laag, maar op het einde vertoont deze een plotse stijging. Van dit merkwaardig drukprofiel van dex-HEMA wordt gebruik gemaakt om de lipid coating plots te doen barsten, waardoor ingesloten geneesmiddelen in één keer worden vrijgesteld. Om te vermijden dat ingesloten moleculen te snel vrijgesteld

worden door defecten in het membraan, is het nodig om voldoende stevige membranen te gebruiken. Vaak is de druk die bereikt wordt door degraderende dex-HEMA hydrogels onvoldoende. Het is bekend dat PEG hogere osmotische drukken vertoont in oplossing dan dextraan. Vandaar onze interesse in PEG: wanneer hogere osmotische drukken bereikt worden, kunnen ook sterkere membranen gebroken worden. Naar analogie met dex-HEMA wordt ook PEG voorzien van een polymerizeerbare groep wat resulteert in hydroxyethyl methacrylaat PEG (PEG-HEMA).

Om het zweldruk profiel op te stellen van PEG-HEMA werd een reeds eerder gepubliceerde manier gebruikt om degraderende gels na te bootsen met behulp van niet gedegradeerde PEG-HEMA gels die verschillende hoeveelheden vrij PEG (afbraakproduct) bevatten. Op deze manier werd aangetoond dat PEG-HEMA een gelijkaardig osmotisch drukprofiel vertoont als dex-HEMA, maar veel hogere “drukpieken” vertoont dan dextraan.

Om een beter inzicht te krijgen in de bijdrage van vrije ketens aan de osmotische druk zijn we nagegaan wat is van het effect van het moleculair gewicht van de vrije ketens in dex-HEMA en PEG-HEMA hydrogels. We ontdekten dat het verschil tussen de bijdrage van netwerk ketens en vrije ketens tot de osmotische druk afneemt als het moleculair gewicht van de vrije ketens afneemt. Of eenvoudiger gezegd: vrije ketens met een hoog moleculair gewicht gedragen zich als netwerk ketens.

In **hoofdstuk zes** worden zowel microscopische als nanoscopische hydrogel partikels gemaakt uitgaande van PEG. Er werden twee nieuwe polymerizeerbare PEG moleculen aangemaakt: enerzijds PEG-lactaat-HEMA, dat dankzij zijn lactaat spacer sneller afbreekt dan PEG en anderzijds stervormige PEG-HEMA (✳-PEG-HEMA). Deze “ster” heeft 8 armen en aan het uiteinde van elke arm wordt een hydroxyethyl methacrylaat groep gekoppeld. Op deze manier bevat ✳-PEG-HEMA meer polymerizeerbare groepen en breken de overeenkomstige hydrogels beduidend trager af in vergelijking met PEG-HEMA.

Via emulsiepolymerizatie is het mogelijk om microgels te maken van zowel PEG-HEMA, PEG-lac-HEMA en ✳-PEG-HEMA. Wanneer we ze co-polymerizeren met een positief of negatief geladen methacrylaat kunnen we ze bovendien voorzien van een lipid coating. De lipid coating is gebaseerd op elektrostatische interactie tussen de geladen microgels en het tegenovergesteld geladen lipid membraan. De lipid gecoate microgels konden gevisualiseerd worden via confocale microscopie. Om de mogelijkheid na te gaan om deze (lipid gecoate) PEG microgels te gebruiken als afgiftesysteem voor geneesmiddelen, werd het vrijstellingsprofiel van FITC-PEG geëvalueerd. Ingesloten moleculen worden het snelst vrijgesteld door microgels gemaakt uit PEG-lac-HEMA: reeds na enkele uren is de vrijstelling voltooid. ✳-PEG-HEMA microgels daarentegen geven hun inhoud het traagste af: de vrijstelling van FITC-PEG duurt een maand. PEG-HEMA stelt zijn inhoud vrij gedurende een drietal weken. Hoewel de lipidcoating blijkbaar nauwelijks invloed heeft op het

vrijstellingsprofiel, zou het gebruik ervan wel handig kunnen zijn om de microgels te voorzien van een functionele coating zoals beschreven in hoofdstuk 4.

Naar analogie met dex-HEMA nanogels beschreven in hoofdstukken 2 tot 4, worden ook PEG-HEMA nanogels gemaakt op dezelfde methode: een lipid film wordt gehydrateerd met een PEG-HEMA oplossing en daarna gepolymerizeerd met UV. De degradatie van PEG-HEMA nanogels verloopt veel trager, wat we ook reeds vaststelden op macroscopische schaal in hoofdstuk 5: volledige degradatie duurt 35 dagen. Ingesloten BSA wordt sneller vrijgesteld dan verwacht: na 7 dagen is alle ingesloten BSA vrijgesteld.

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Aanmaak pH gevoelige lipiden (Prof. D. Thompson, Purdue University, West Lafayette, USA). (14-29 januari 2006)

Deelname aan congressen

Niet actieve deelname:

13 december 2002: **Belgian-Dutch Biofarmacy Day in Leuven**

19-23 Juli 2003: **30th Annual Meeting of the Controlled Release Society in Glasgow, Scotland**

Actieve deelname:

6-9 Maart 2004: "Lipid coating of degrading hydrogel microparticles for drug delivery" (poster)
Particles 2004 in Orlando, Florida, USA

3 december 2004: "Synthesis and characterization of biodegradable nanogels" (poster)
Belgian-Dutch Biofarmacy Day in Oss, Nederland

20 mei 2005: "Polysaccharide Nanogels with tunable Degradation properties" (lezing)
Belgian-Dutch Biofarmacy Day in Gent

7 maart 2006: "New Drug Delivery Opportunities for Micro- and Nanogels?" (lezing)
Cell culture and in vitro models for drug absorption and delivery in Saarbrucken, Duitsland

5-7 april 2006: "On the synthesis and characterization of biodegradable dextran nanogels with tunable degradation properties" (poster)
9th European Symposium on Controlled Drug Delivery in Noordwijk Aan Zee, Nederland

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